The Journal of Laboratory and Clinical Medicine

VOL. IX ST. LOUIS, MO., JUNE, 1924 No. 9

CLINICAL AND EXPERIMENTAL

OBSERVATIONS ON THE PHYSIOLOGICAL ASSAY OF INSULIN*

BY J. J. R. MACLEOD, AND M. D. ORR, TORONTO, CANADA

DURING the year or so which has elapsed since insulin has been used in the treatment of diabetes, its assay has depended upon the method originally described by Banting, Best, Collip, Macleod and Noble.[1] This consists in measuring the extent to which the blood sugar becomes reduced in a normal rabbit within a period of three to four hours, one unit being considered as that amount which brings it to the convulsive level (0.045 per cent) in this time. It was early recognized that this method is not strictly accurate even when precautions are taken to use animals of uniform size and breed and in which the amount of glycogen has been reduced by a short preliminary period of starvation (McCormick, Macleod, Noble and O'Brien[2]). This has made it necessary to use large numbers of animals for the assay of each batch of insulin, and in order to avoid the necessity of having to determine the blood sugar, several investigators have based the assay on the incidence of the well-characterized symptoms which supervene when the blood sugar attains a certain level. This method has been used on rabbits in the Eli Lilly laboratories at Indianapolis, varying quantities of a given preparation of insulin being injected into a number of animals and the unitage calculated from the proportion of these which develop convulsive symptoms within a certain time. It is evident, however, if the development of symptoms is to form the sole basis for the assay, that smaller animals should be used and mice have been chosen for this purpose by A. Krogh, H. H. Dale and J. H. Burn and D. Fraser.[3] Krogh found when these animals after injection with insulin, are kept under ordinary laboratory conditions that the most constant result is a profound fall in body temperature and weakness, with convulsive symptoms only occasionally, but that when the fall in temperature is prevented by

*From the laboratory of the Insulin Committee, University of Toronto.

keeping the mice in an incubator at about 28° C., characteristic symptoms supervene, paralysis of the legs, convulsions and coma, which can be antidoted by injections of glucose. By also using precautions to standardize the diet and to use animals of similar age (varying from 16 to 20 grams in weight) he has evolved a method of assay in which one mouse unit is considered as the amount which will cause definite symptoms in 50 per cent of the injected animals. A. Krogh states that the mouse compared on a body weight basis is about five times more sensitive to insulin than the rabbit so that 500 mouse doses (in a 20 gram mouse) would correspond to one rabbit unit. H. H. Dale on the other hand, finds mice and rabbits to be equally sensitive to insulin (on a body weight basis). If this method can be satisfactorily standardized, it is likely to become a useful one for practical purposes, but it is questionable, for reasons which will be given in this paper, whether the incidence of convulsive symptoms can form a safe basis for accurate assay.

Another basis for assay has been investigated by F. N. Allan[4]; namely, determination of the glucose equivalent of insulin when given to completely depancreatized dogs kept on a constant diet of meat and cane sugar. When the amount of insulin, given daily, is varied, the change occurring in the sugar balance will indicate the numer of grams of glucose which a given quantity of insulin can cause to be metabolized. It has been found that this method yields constant results provided the sugar balance is at the same level. The glucose equivalent of a unit of insulin is not, however, a constant quantity, but is dependent on certain factors. One of these is the size of the dose of insulin, the glucose equivalent per unit being much greater with small doses, than with large. Thus, in a depancreatized dog fed daily with 600 g. meat and 100 g. cane sugar, the glucose equivalent per unit was 5.8 when 20 (clinical) units were given daily; 4.0 with 24 units; 3.5 with 32 units and 3.1 with 40 units. Another factor is the carbohydrate intake; with the same dose of insulin the glucose equivalent per unit is greater when the intake of carbohydrate is high than when it is low. Thus, in the same dog while receiving daily 20 units of insulin along with varying amounts of cane sugar, the average glucose equivalents were found to be 3.7; 5.8 and 7.6 with daily allowances of 50, 100 and 150 grams cane sugar respectively.

One of us with G. S. Eadie[5] has also investigated the possibility of basing the assay on the power of insulin to prevent the development of the hyperglycemia which is produced in rabbits either by injecting measured quantities of glucose or by injecting epinephrin. Since the results showed about the same degree of variation as those obtained with the original method, based on the lowering of the blood sugar below the normal level, further attention has not been given to these methods recently.

The present paper deals with certain facts which have come to light in carrying out assays by the original method. These assays have been made on samples taken from each of the batches of insulin that have been distributed for clinical use in Canada and the United States.

METHOD

In the method as originally defined[1] there are several obvious sources of inaccuracy. Thus, it is impossible to know from a few determinations of blood sugar, at intervals following the injection of insulin that the lowest result observed represents the lowest level to which the blood sugar fell. In the original method of calculation also no allowance is made for the normal blood sugar of the animal, preceding the injection, or for the duration of the hypoglycemia.

In order to allow for these factors and also for the varying weights of the animals used we have used the following equation:

Units per c.c. $= \frac{a}{b} \times \frac{w}{c} \times \frac{1}{2}$ in which **a** represents the difference between the percentage of blood sugar before injection and the average of the percentages found in 1½, 3 and 5 hours following the injection*; **b,** the difference between the normal blood sugar and 0.045 per cent (this being the level at which convulsions are likely to supervene); **w,** the weight of the animal in kg.; **c,** the number of cubic centimeters of insulin injected. The product of the equation is multiplied by ½ since the original unit was based on the effect produced on a rabbit weighing 2 kg.

Acting on the advice of our clinical associates, we have more recently adopted a unit which is one-third that of the original unit as defined above, the reason given being that with the smaller unit it would be unnecessary in clinical practice to use fractions for the treatment of mild cases. This clinical unit, as it is called, is therefore obtained from the equation: $\frac{a}{b} \times \frac{w}{c} \times 1.5$. To prevent confusion the original unit is called the "physiological unit" and the new one "the clinical unit." It is, of course, realized that this equation is empirical and that it fails to take into account several well-known features of the action of insulin. Thus, with regard to the values of **a** and **c** it is certain that **a** does not increase in direct proportion to the dose of insulin (**c**). On the contrary, as the dose (**c**) increases the extent to which the blood sugar is lowered (**a**) with each unit of increase in dose becomes progressively less and less, forming a curve which is probably similar in contour to that observed by F. N. Allan for the glucose equivalents of insulin. We have endeavored to obtain data from which such a curve could be drawn for the hypoglycemic action of insulin but, as will be shown later, this has not been possible because of the great variability in the effect produced even when the same animal is injected at intervals of several days with the same amounts of insulin. Until the exact equation of this curve is determined, the only procedure which appears at all likely to yield constant values is to disregard those results which are obtained with either very small or very large doses of insulin, for in the former case the values of **a** would be relatively too great and in the latter too small. Since

*These periods are chosen since in five hours the blood sugar, with a moderate dose of insulin, has usually returned to about the normal level and it is usually at its minimum in about one and one-half hours. More recent trials in which intervals of forty-five minutes, two hours and five hours were used did not yield more satisfactory results.

the possible range through which the blood sugar may fall in normal rabbits as a result of the injection of one unit of insulin is not more than about 65 mg. per cent and since the very smallest effective dose of insulin may lower the blood sugar through half this range, and twice this dose cause a further reduction to say one-half of the remainder it is obvious that the different values for **a** with larger doses (**c**) must become so small as to fall within the experimental error of the method. In order to increase the range through which the blood sugar would be caused to fall by insulin one of us, with G. S. Eadie, attempted to increase the accuracy of assay by raising the blood sugar either by injecting glucose or by stimulating its production endogenously by injecting epinephrin into glycogen-rich animals. In the latter experiments an equation was found expressing the relation between the dose of insulin and the blood-sugar level, and it is possible that more extended observations by this method might show it to be a useful one for practical purposes. Until more data have been collected, however, we must arbitrarily take for **a** those results obtained with doses of insulin which lower the blood sugar to a distance which is more than half way between the normal and the convulsive level.

There is some evidence that the duration of the effect of insulin depends within certain limits on its concentration in the solution injected; thus, one unit diluted to 2 c.c. has a more lasting effect than when diluted to 1 c.c. so that the average level of the blood sugar will be lower and **a** consequently greater. The difference is probably related to the rate of absorption of insulin from the seat of injection. This possible source of inaccuracy has been controlled by using solutions of equal dilution.

The value, **b,** is used merely to make the equation conform with the definition.

In using the value, **w,** the assumption is made that the effect of insulin is proportional to the body weight, which is probably not the case, the small animal reacting relatively more acutely than the larger, because the metabolic processes are more intense. As already mentioned, A. Krogh believes that mice are about five times more sensitive than rabbits, allowing for the differences in body weight, but Dale considers them about equally sensitive. The comparison is, of course, based on the incidence of symptoms.

Technic.—In order to diminish as far as possible inaccuracy of assay due to the sources of error indicated above, the following *methods of procedure* have been adopted at varying stages of the work: (1) Normal blood is removed from the ear veins of three rabbits of about the same weights (each previously starved for 24 hours) and into one of the animals is injected an amount of insulin expected to correspond to one physiological, or three clinical units. Into a second rabbit an amount of insulin from 25 to 50 per cent less than one unit is injected and into a third an amount of 25 to 50 per cent greater; blood sugars are determined in one and one-half, three and five hours, and the results calculated by use of the above equation. If the assays vary by more than 25 per cent between extremes they are repeated on another group of three rabbits using this time for the first rabbit the amount of insulin which gave the highest value in the first test

and 25 to 50 per cent more or less for the second and third rabbits, respectively. If the results still disagree within the above limits, they are repeated on the same principle until satisfactory values are obtained. and it is on the average of these that the final assay is based. (2) A modification of the above method and that which is being tested at present is to choose three doses of insulin which lie between the limits of one physiological unit and one-half unit and inject three rabbits with each of these doses. Provided the results of the three rabbits in each group, as calculated from the formula, agree within 25 per cent between extremes, they are averaged and the average of the three values thus obtained is taken as the final assay. If one result in any one of the groups disagrees from the others by more than 25 per cent, the corresponding dose of insulin is reinjected into three other rabbits and this is continued until there is satisfactory agreement among the individual results of each group. If the three averages do not fall within the limits of 25 per cent between extremes the whole assay is repeated using nine rabbits. (3) Into one of the nine animals is injected an amount of insulin expected to correspond to one physiological or three clinical units and into a second, an amount of 50 per cent greater. The other seven rabbits are injected with amounts varying from slightly below one physiological unit to one half a unit. Blood sugars are determined at intervals of one and one-half, three and five hours and the results calculated by using the formula. The highest results which agree within 25 per cent are averaged and the average is taken as the assay, provided at least five of the results fall within this range. If not so, the material is retested until the majority of the results correspond within the above limits.

In the second and third of these methods the attempt is made to use doses which fall in the middle portion of the curve expressing the relationship between effect and dosage. This demands, of course, a knowledge of the approximate strength of the preparation of insulin and to gain it a preliminary assay may be required. In practice, however, the strength of the preparation

TABLE I

INSULIN NUMBER 741141

WEIGHT IN Kg.	DOSE PER 2 Kg.	DOSE GIVEN	BLOOD SUGAR NORMAL	BLOOD SUGAR 1½ HR.	BLOOD SUGAR 3 HR.	BLOOD SUGAR 5 HR.	AVERAGE	(A)	(B)	$\frac{A}{B} \times \frac{W}{C}$
1.90	.200	.190	.104	.058	.056	.061	.058	.046	.059	7.80
1.75	.150	.131	.112	.050	.059	.100	.070	.042	.067	8.37
2.38	.120	.143	.122	.051	.040	.121	.071	.051	.077	11.03
2.07	.100	.103	.117	.063	.078	.104	.082	.035	.072	9.77
1.75	.090	.079	.114	.050	.064	.114	.076	.038	.069	12.20
2.22	.080	.089	.117	.077	.081	.101	.086	.031	.072	10.74
2.28	.070	.080	.118	.043	.048	.067	.053	.065	.073	25.37
2.00	.060	.060	.117	.061	.104	.116	.094	.023	.072	10.64
2.17	.080	.087	.095	.043	.063	.102	.069	.026	.050	12.97
1.89	.100	.094	.117	.046	.064	.119	.076	.041	.072	11.45
1.73	.080	.069	.119	.069	.080	.126	.092	.027	.074	9.15
1.91	.070	.067	.109	.069	.096	.114	.093	.016	.064	7.12
1.84	.120	.110	.100	.056	.067	.105	.076	.024	.055	7.30

Assay $\frac{a}{b} \times \frac{W}{c} \times 1.5 = 16$

The underlined figures are those used for the assay by method 3 (four of the doses being given in duplicate) and give 16.8.

can usually be calculated with tolerable accuracy either from the amount of the final precipitate of insulin (either that obtained by precipitation at the isoelectric point or by preparing the so-called hydrochloride) or from the nitrogen content of the solution, so that preliminary assay is not necessary. It has been found that for 20 unit material the most satisfactory results are obtained with 0.125 c.c., 0.100 c.c., and 0.075 c.c. injected into a rabbit weighing 2 kg. These amounts are diluted to 1 c.c., 0.8 c.c. and 0.6 c.c., respectively, so that the concentrations of the injected fluids may correspond. Table I will illustrate the data for an assay computed by the third method described above.

ACCURACY OF ASSAYS

Assays by these methods have been carried out on over 300 batches of insulin with every precaution as to the condition of the animals employed, and although the results have proved to be of practical value they do not agree as closely as could be desired. That a certain degree of variation is inevitable in the method is indicated by the fact that even when equal doses of the same insulin are given to rabbits of practically the same weights considerable variation may be obtained in the assays. This is shown in Table II.

TABLE II

1. COMPARISON OF THE EFFECTS OF SIMILAR DOSES OF THE SAME PREPARATIONS OF INSULIN GIVEN TO RABBITS OF EQUAL WEIGHT (WTIHIN 100 GMS.)

NO. OF INSULIN PREPARATION	WT. GM.	DOSE GIVEN PER 2000 GM. C.C.		VALUE OF EQUATION $\frac{A}{B} \times \frac{W}{C}$	NO. OF INSULIN PREPARATION	WT. GMS.	DOSE GIVEN PER 2000 GM. C.C.		VALUE OF EQUATION $\frac{A}{B} \times \frac{W}{C}$
1502	1730	.108	.125	19.5	B. 1475	1450	.090	.125	8.24
	1850	.116	.125	15.7		1450	.090	.125	12.66
218	1820	.136	.150	4.86	205	1680	.139	.166	5.7
	1970	.148	.150	6.83		1650	.136	.166	8.8
	1920	.240	.250	6.83					
	1830	.228	.250	5.15	B. 1497	1500	.094	.125	7.4
						1500	.094	.125	7.0
B. 1496	1540	.096	.125	8.02					
	1650	.103	.125	3.91	737	1550	.194	.250	6.2
	1790	.179	.200	7.70		1560	.195	.250	4.5
	1800	.180	.200	6.71					
	1820	.182	.200	4.20					
B. 1466	1860	.139	.150	10.65					
	1820	.136	.150	7.37					

It is to be expected therefore that the same dose per kilo body weight given to rabbits of different weights will also give variable results but it is significant that the degree of variation is no greater than with uniform rabbits. This is shown in Table III.

No estimate can be made from these figures of the accuracy of the method, since they are selected on the basis of body weight instead of on that of the most closely corresponding of the assays. In Table IV certain assays are selected to show the degree of accuracy that may be expected, using the first

TABLE III

EFFECTS OF THE SAME DOSES (C.C.) PER KG. BODY WEIGHT ON RABBITS OF DIFFERENT WEIGHTS

NO.	WEIGHT	DOSE GIVEN C.C.	DOSE PER 2000 GM.	VALUE OF $\frac{A}{B} \times \frac{W}{C}$	REMARKS
B. 1447	1460	0.091	0.125	5.4	
	2030	0.125	0.125	5.3	
B. 1456	2670	0.166	0.125	13.8	Convulsions in 1 hr. 19 mins.
	1800	0.112	0.125	11.6	
201	1350	0.168	0.250	4.1	
	1980	0.247	0.250	6.2	
B. 1466	1680	0.080	.100	8.9	
	1920	0.096	.100	15.6	
B. 1476	1600	0.100	.125	11.8	
	1900	0.118	.125		Convulsion in 52 mins.
	2550	0.159	.125	9.13	
210	1450	0.091	.125	14.1	
	2190	0.136	.125	10.8	
210	1540	0.192	.25	9.5	
	1900	0.237	.25		Convulsions in 1 hr. 29 mins.
211	1500	0.187	.25	8.81	Convulsions in 1 hr. 37 mins.
	2070	0.258	.25	8.16	Convulsions in 1 hr. 35 mins.
B. 1484	1480	.092	.125	11.8	
	1700	0.106	.125	17.6	Convulsions in 1 hr. 12 mins.
213-2	1360	0.085	.125	13.2	
	1650	0.103	.125	7.2	
	1470	0.183	.250	6.67	Convulsions in 1 hr. 26 mins.
	2150	0.269	.250	12.8	
B. 1496	1470	0.128	.175	5.3	
	1870	0.163	.175	5.13	

TABLE IV

ASSAYS OF INSULIN. MOST OF THE ASSAYS ON EACH BATCH OF INSULIN WERE DONE ON DIFFERENT DAYS

Method 1

Insulin No.	c.c. per 2000 gms.	$\frac{a}{b} \times \frac{w}{c}$	Average
221	.200	8.26	
	.150	8.55	8.4
	.250	5.14*	
	.125	9.50	
	.150	5.75*	8.6
	.175	7.64	
218	.150	4.86*	
	.200	7.81	6.9
	.250	6.03	
	.175	4.87*	
	.200	6.75	6.8
	.250	6.83	
	.125	7.64	
	.150	6.83	6.9
	.225	6.61	
	.300	6.46	
710	.500	3.87	
	.250	7.49	
	.750	2.50	
	.300	7.40*	
	.200	5.10	5.9
	.150	6.80	
	.250	5.40	
	.175	5.10	5.3
	.125	5.50	

*Figures marked with * not included in averages.

TABLE IV

Method 3

Insulin No.	c.c. per 2 Kgm.	$\frac{a}{b} \times \frac{w}{c}$	Assay Reported	Remarks
250	.120	14.6		
	.100	14.1		
	.130	11.7		
	.140	8.0		
	.070	15.0	22	
	.080	17.9		
	.110	15.3		
	.150	13.5		
	.120	11.7		
250-3	.250	6.9		
	.200	7.7		
	.150	8.4		
	.170	7.1		250-3 was made from 250
	.200	7.0	11	and was calculated to
	.180	7.8		be 10 unit material.
	.190	7.1		
	.250	4.64		
	.300	4.9		
-150	.150	Bad convuls.		
	.130	12.6		
	.100	14.4		
	.090	12.8		
	.080	10.8	20	
	.070	13.5		
	.080	16.5		
	.090	Bad convuls.		
	.100	12.6		
-149	.100	7.8		
	.170	6.7		
	.200	3.7		
	.160	6.5	10	-149 was 1:1 dilution of
	.180	7.2		-150.
	.170	6.8		
	.150	10.4		
	.170	6.5		
	.250	6.1		
-132	.100	12.5		
	.080	12.9		
	.110	convuls.		
	.120	9.4		
	.130	7.3		
	.140	9.4	16.1	Tested Oct. 15, 1923.
	.150	10.9		
	.100	16.4		
	.100	9.4		
-132	.120	10.5		
	.140	9.9		
	.130	5.9		
	.120	10.0	15.8	Tested Oct. 17, 1923.
	.110	7.9		
	.100	convuls		
	.080	11.6		
	.120	9.8		

and third methods described on pp. 594 and 595 but, of course, averages from many more would be necessary to indicate this correctly.* The observations are grouped, those in each group having been made on the same days, and the averages are those upon which the assays were based (the asterisked figures not being included).

COMPARISON OF ASSAYS BASED ON BLOOD SUGAR WITH THOSE BASED ON THE INCIDENCE OF CONVULSIONS

As already pointed out convulsive symptoms are first observed when the blood sugar is somewhere about 0.045 per cent, (i.e., between 0.040 and 0.050).

In five rabbits out of 971 symptoms were observed before the blood sugar fell below 0.050 per cent. Thus, in the case of insulin 221 the blood sugars in one and one-half, three and five hours were 0.050, 0.054, and 0.097 per cent, respectively, and convulsions occurred in two hours twenty minutes; in that of 223, convulsions occurred in three hours when the blood sugar was 0.058 per cent. It is most likely that these apparent exceptions are due to the bloods having been removed either before or after the percentage of sugar had reached its lowest level. When the blood sugar is falling rapidly it is obvious that a sample of blood removed a minute or so before convulsions may contain considerably more sugar than when these occur. On the other hand, in one removed after the convulsion the blood sugar may have already been partially restored as a result of the more rapid glycogenolysis which as Miss O'Brien has found, is set up by the convulsions, especially when glycogen still remains in the liver. In such cases were the incidence of convulsions alone depended upon, the power of insulin to lower the blood sugar would be assayed at too low a value.

Much more frequently the blood sugar is found to be considerably below 0.040 per cent at the time convulsions are observed to occur, and they may fail to be observed at all although one or more of the three blood sugars is decidedly below that level. Thus, out of a total of 971 rabbits injected with insulin no symptoms are recorded in 158 in which one or more of the blood sugars was decidedly below 0.040 per cent. The correct statement of the relationship between symptoms and the blood-sugar level is therefore that they are likely to occur after the percentage has fallen below 0.045 per cent, but they remain absent at considerably lower levels. It is altogether probable that the greater frequency of symptoms at the higher levels, as observed by us in earlier work, is due to the fact that the rabbits were not subjected to a preliminary period of starvation, so that they contained considerable reserves of glycogen. By gradual mobilization of glucose from these reserves, following the initial fall in blood sugar, a further tendency to the development of hypoglycemia would be partially counteracted although the blood sugar would remain at a level (0.040 – 0.050 per cent) at which toxic effects ultimately become developed. In starved animals on the other hand the blood sugar continues its rapid descent and goes below the level at which symptoms may appear because the toxic condition which, by its action on the nerve centers, is responsible for

*A statistical treatment of all assays will be published shortly.

them, has not had time to become developed. It is therefore especially when large doses of insulin are given that the blood sugar is likely to be much below 0.040 per cent when symptoms supervene. Some rabbits are decidedly more susceptible to convulsions as a result of hypoglycemia, than others. Thus, we have frequently observed animals that regularly remained free of symptoms even when the blood sugar fell well below 0.04 per cent.

Whatever may be the explanation for the irregularity between the onset of symptoms and the level of blood sugar it is clear that assays based on the former cannot represent with accuracy the hypoglycemic potency of the preparations, and since it is this that we desire to know for clinical purposes it is desirable for the present at least that the assays be based on the method recommended in this paper.

Another objection to using the incidence of convulsions as the basis of assay is that no estimate is made of the duration of the hypoglycemic effect. As pointed out elsewhere, this duration depends very largely on the amount of glycogen stored in the liver and muscles of the animal, and it is possible that if we could be certain that all the animals used in the assay were free of glycogen, or contained exactly the same amounts, the same doses of the same insulin would cause hypoglycemia of equal duration. But even if this could be attained it would not necessarily follow that with different preparations of insulin the hypoglycemia is always of the same duration. It is possible, not only that the rate of absorption may vary but also that the presence of extractives from the pancreas may antagonize or delay the action of insulin. In this connection it is of importance to remember that one of us[6] has observed that extracts of the zymogenous tissue in certain of the bony fishes (Lophius) often cause a slight but distinct increase in blood sugar and that J. B. Collip[7] has found that insulin prepared by the usual methods from yeast, clams, etc.,

TABLE V

COMPARISON OF RABBIT UNITS CALCULATED FROM THE SMALLEST AMOUNT FOUND TO CAUSE CONVULSIONS AND THOSE CALCULATED FROM FORMULA

1 NO.	2 SMALLEST AMOUNT CAUSING CONVULSIONS (C.C. PER 2 KG.)	3 ORIGINAL UNITS ESTIMATED FROM CONVULSIONS	4 ORIGINAL UNITS ESTIMATED BY FORMULA (DIVIDED BY 2)
B. 1466	0.375	2.6	5
B. 1475	0.125	8	7
213–2	0.166	6	4.3
1502	0.100	10	6.5
1504	0.150	7	4.8
223	0.150	7	5.5
710	0.300	3.5	3.2
215	0.125	8	4
721	0.175	6	5
216	0.250	4	3.5
1498	0.175	6	4.6
213	0.250	4	5
197–2	0.200	5	4.7
201–2	0.333	3	1.3
205–2	0.250	4	4.6
206	0.200	5	6.3
B. 1440	0.250	4	3.1
B. 1446	0.375	2.6	3
194	0.250	4	4.25

may not cause a marked degree of hypoglycemia until many hours after injection. It has also been our impression, although the truth of it has not been investigated, that impure preparations of insulin have a more prolonged hypoglycemic effect than those that are highly purified.

Within considerable limits the incidence of convulsions does not depend on body weight.

In Table V the number of physiological units per cubic centimeter of various samples of insulin have been computed; in column 3 from the smallest dose which caused convulsions and in column 4 from the average lowering of blood sugar, by the formula $\frac{a}{b} \times \frac{w}{c} \times \frac{1}{2}$. It will be seen that the assay is usually considerably higher when based on the incidence of convulsions than on the degree of hypoglycemia. In about one-half of the cases chosen the difference was more than 25 per cent. Had the basis which is used by Dr. Clowes of the Eli Lilly Co. in the case of rabbits and by A. Krogh in the case of mice been chosen, namely, the smallest amount of insulin which would produce convulsions in a certain percentage of the animals, the values obtained in column 3 would probably have been lower and the discrepancy between the results of the two assays therefore less. It is also probable that the occasional wide discrepancies seen in the above results (as in Nos. B1466, 1502, 215) would have been eliminated. Notwithstanding these criticisms, it is interesting that the assays by the two methods are usually not very far apart, and it is possible that future work may indicate how the physiological conditions of the animals may be standardized so as to ensure a closer correspondence between the blood-sugar level and the incidence of symptoms.

TABLE VI

INSULIN 284

WEIGHT IN KGMS.	DOSE PER 2 KGMS.	BLOOD SUGAR				CONVULSIONS
		NORMAL	1½ HOURS	3 HOURS	5 HOURS	
2.05	.150	.114	.035	.028	.037	
2.01	.100	.109	.052	.050	.074	
1.84	.090	.117	.033	.026	.074	
1.93	.080	.093	.056	.035	.040	
2.04	.070	.105	.024	.030	.090	1 hr. 58
1.90	.060	.111	.033	.053	.097	
1.84	.070	.105	.038	.046	.074	
1.59	.070	.110	.060	.070	.111	
2.03	.070	.105	.033	.020	.070	2 hr. 12
1.86	.070	.110	.052	.028	.058	
1.90	.100	.101	.042	.035	.070	
1.87	.100	.095	.061	.054	.077	
2.14	.070	.092	.038	.040	.083	
1.94	.070	.107	.054	.054	.060	

In a sample of insulin recently assayed and shown in Table VI it is seen that of fourteen rabbits used only two showed convulsions, although in ten one or more of the blood sugars was below 0.040 per cent. If convulsions had been used as a basis of assays in this case it is doubtful whether the hypoglycemic potency of the sample could have been shown. The assay was 38 units.

ASSAYS BASED ON THE RATE OF DEVELOPMENT OF THE INITIAL STAGES OF HYPOGLYCEMIA WITH VARYING SMALL DOSES OF INSULIN

As has been pointed out elsewhere[2] a characteristic feature of the action of insulin when given in moderate amounts is that the initial fall in blood sugar is practically the same, although the dose may vary considerably. The fall in blood sugar which occurs during the first half hour following the injection of an amount of insulin that is sufficient to cause convulsions within an hour or so in a starved animal will be practically the same as that following a much smaller amount given to a well-fed one. This "all or nothing" effect would seem to indicate that the reduction in the percentage of blood sugar following insulin does not cause a compensatory increase in the output of sugar from the glycogen stores of the liver until a certain low level, or threshold, has been reached; or, if it does so, that the amount of sugar thus added to the blood is insignificant in comparison with the amount which is disappearing. Later when the effect of insulin begins to pass off, perhaps because of excretion of the excess of urine, glycogenolysis comes to be able to cope with the hypoglycemia, the fall becomes less steep and, finally, recovery sets in. These considerations indicate the possibility that the dosage of insulin might be determined by measurement of the steepness and the *depth of the fall in blood sugar which occurs during the first half hour after*

TABLE VII

NO.	CONDITION OF ANIMAL	DOSE OF INSULIN (C.C. PER Kg.)	NORMAL (PER CENT)	BLOOD SUGAR AFTER ½ HR. (PER CENT)	BLOOD SUGAR FALL (MG.)	
4	Starved & adrenalin	0.01	.093	.024	69	
		0.015	.112	.065	47	
		0.02	.101	.046	55	
		0.03	.082	.046	32	
		0.04	.080	.036	44	
1	Starved	0.01	0.109	0.096	(13)*	
		0.01	0.104	0.083	21	
		0.02	0.142†	0.094	48	
		0.02	0.128†	0.085	43	
		0.03	0.105	0.061	44	
		0.04	0.106	0.052	54	
		0.04	0.099	0.046	53	
2	Starved	0.01	0.116	0.077	39	‡
		0.02	0.123	0.088	35	
		0.03	0.125	0.043	82	
		0.04	0.138	0.080	58	
3	Starved	0.05	0.100	0.070	30	
		0.06	0.104	0.031	73	
		0.1	0.117	0.080	37	
		0.1	0.108	0.047	61	
		0.2	0.108	0.047	61	
5	Starved	0.005	0.122	0.095	27	
		0.0075	0.109	0.083	26	
		0.010	0.105	0.059	46	

*Slight rise to start.
†Samples taken in 10 and 40 mins. after injection.
‡All samples taken in 10 and 40 mins. after injection.

giving varying, very minute, amounts to deglycogenated animals. This has been done in the following experiments shown in Table VII.

In order that no error due to inequality in the rate of absorption might occur the injections were made intravenously by using a very accurate syringe, the varying quantities of insulin being obtained by dilution with water at a pH below 4. Most of the rabbits were starved for one day preceding the injections, but in some they were rendered aglycogenic by longer starvation coupled with injections of epinephrin.* In some of the observations the readings were taken in ten and forty minutes following the injection instead of after thirty minutes because a slight rise in blood sugar occurred in the first ten minutes.

It is evident that the rate of fall cannot be used as a basis of assay although in all of the observations with the exception of those made on animals that had been rendered aglycogenic by epinephrin (No. 4) the smaller doses caused decidedly less of a fall than the larger. The most satisfactory ob-

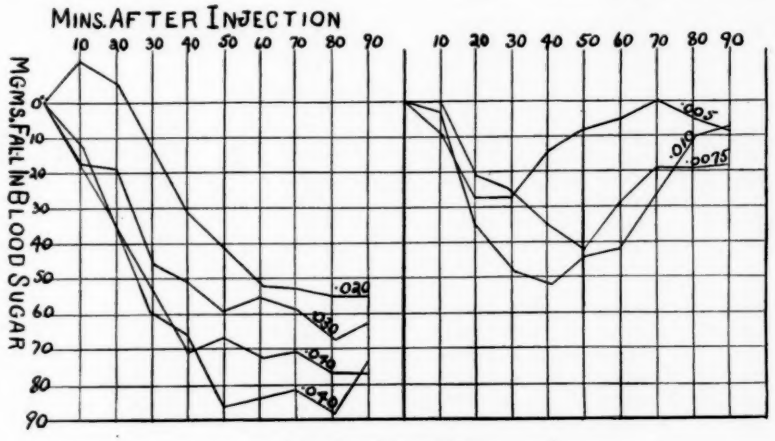


Fig. 1.—See context.

servation is that of No. 1 the results of which are therefore given in greater detail in curve form (Fig. 1). In one of the cases in which 0.02 c.c. was injected the normal blood sugar was above the permissible level, and neither this curve, nor that of one of the curves following 0.01 c.c., are satisfactorily regular but the results are nevertheless included. In this case a closer parallelism between dosage and effect is evident in one hour after the injection than it is in thirty minutes, but this is not the case as a rule. Thus in No. 5 in which the lowest dose was just sufficient to cause a slight temporary decline in blood sugar the parallelism is less evident after the longer interval. It is clear therefore that the assay of insulin cannot be based on the steepness of the initial fall in blood sugar.

ASSAYS BASED ON OBSERVATIONS ON THE SAME RABBITS AT INTERVALS

The results given in Tables II, III, and IV are taken from a long series of assays made on different stock rabbits. Although all the animals were

*Work recently completed along with J. Markowitz has shown, in confirmation of that of L. Pollak, that epinephrin cannot be depended on to render the liver free of glycogen.

TABLE VIII

REGISTERED RABBITS

Each rabbit received the same dose per 2 Kg. body weight in each of its tests. The different rabbits have not all been given the same dose per 2 Kg.

DATE	DOSE OF INSULIN PER 2 Kg. BODY WEIGHT 0.1 C.C.				DEVIATION FROM AVERAGE
	GMS. WEIGHT	NORMAL	1½, 3 & 5 HR. AVERAGE	NORMAL AVERAGE "A"	
No. 1					
29/11/23	2000	.100	.062	.038	+ 1
4/12/23	2010	.122	.076	.046	+ 9
12/12/23	1960	.093	.068	.025	−12
7/ 1/24	2040	(.122	.050	.072)	
12/ 1/24	2050	.110	.079	.031	− 6
28/ 1/24	1820	.126	.080	.046	+ 9
			Average	37	
No. 2					
29/11/23	2000	.112	.076	.036	+ 3
4/12/23	1990	.114	.080	.034	+ 1
12/12/23	2000	.101	.075	.026	− 7
7/ 1/24	2050	.110	.074	.036	+ 3
12/ 1/24	2090	.106	.079	.027	− 4
28/ 1/24	2040	.106	.067	.039	+ 6
			Average	33	
No. 3					
29/11/23	2000	.114	.061	.053	+ 6
4/12/23	2050	.112	.066	.046	− 1
12/12/23	1940	.104	.074	.030	−17
7/ 1/24	1990	.106	.057	.049	+ 2
14/ 1/24	2050	.109	.047	.062	+15
28/ 1/24	1910	.103	.058	.045	+ 2
			Average	47	
No. 4					
3/12/23	2000	.110	Sugar		
8/12/23	1930	.121	Sugar		
8/ 1/24	2290	.106	.063	.043	− 6
16/ 1/24	2360	.110	.059	.051	+ 2
23/ 1/24	2220	.106	.050	.056	+ 7
29/ 1/24	2340	.114	.068	.046	− 3
			Average	49	
No. 5					
3/12/23	1900	.109	.066	.043	+12
7/12/23	1860	.104	.076	.028	− 3
20/12/23	1860	.100	.061	.039	+ 8
8/ 1/24	2160	.103	.078	.025	− 6
16/ 1/24	2090	.100	.079	.021	−10
22/ 1/24	1890	.102	.066	.036	+ 5
29/ 1/24	2060	.103	.078	.025	− 6
			Average	31	
No. 7					
10/12/23	2040	.096	.073	.023	− 5
17/12/23	2070	.096	.066	.030	+ 2
26/12/23	(2090	.117	.066	.051)	
9/ 1/24	2120	.103	.075	.028	0
17/ 1/24	2200	.097	.069	.028	0
20/ 1/24	2210	.102	.071	.031	+ 3
			Average	28	
No. 8					
10/12/23	2210	.092	.063	.029	− 8
17/ 1/23	2200	.092	.063	.029	− 8
26/ 1/23	(2130	.118	.064	.054)	5
9/ 1/24	2200	.102	.054	.048	+11
17/ 1/24	2300	.095	.057	.038	+ 1
23/ 1/24	2250	.100	.059	.041	+ 4
			Average	37	

TABLE VIII—Cont'd

REGISTERED RABBITS

DATE	DOSE OF INSULIN PER 2 KG. BODY WEIGHT 0.1 C.C.				DEVIATION FROM AVERAGE
	GMS. WEIGHT	NORMAL	1½, 3 & 5 HR. AVERAGE	NORMAL AVERAGE "A"	
No. 9					
10/12/23	2180	.097	.072	.025	
17/12/23	2140	.096	.068	.028	
26/12/23	(2140	.119	.076	.043)	6
9/ 1/24	2040	.107	.068	.039	
17/ 1/24	1980	.105	.073	.032	
23/ 1/24	2000	.106	.066	.040	
30/ 1/24	2040	.113	.063	.050	
			Average	35.5	
No. 10					
11/12/23	1930	.108			
18/12/23	2000	.102	.085	.017	
26/12/23	(1950	.127	.070	.057)	
10/ 1/24	2000	.109	.069	.040	
18/ 1/24	1970	.118	.058	.060)	
24/ 1/24	(2140	.109	.074	.035	
31/ 1/24	2030	.117			
			Average	31	
No. 11					
11/12/23	2180	.117	.089	.028	
18/12/23	2120	.096	.073	.023	
26/12/23	(2100	.130	.067	.063)	
10/ 1/24	2110	.109	.071	.038	
18/ 1/24	2130	.111	.063	.048	
24/ 1/24	2130	.098	.064	.034	
31/ 1/24	2020	.101	Sugar		
			Average	34	
No. 12					
11/12/23	1740	.114	.088	.026	
18/12/23	1790	.108	.076	.032	
26/12/23	(1740	.119	.058	.061)	
10/ 1/24	1760	.095	Sugar		
18/ 1/24	1780	.120	.069	.051	
24/ 1/24	1800	.098	Sugar		
21/ 1/24	1790	.101	Sugar		

fed on the same diet (oats and hay with water) and were starved for twenty-four hours preceding the assays, they cannot be considered as strictly uniform, since they varied in sex, age and breed. It has been observed both by us and by others that different rabbits, although alike in the above mentioned characteristics vary considerably in their susceptibility towards insulin, both with regard to the effect on blood sugar and the incidence to convulsions. This has led us to see whether the same dose of the same insulin administered to the same rabbit on different days would have uniform effects.* Twelve rabbits were chosen, each weighing about 2000 grams although varying in age, sex, and breed. After a preliminary period of several days, during which they were kept under uniform conditions with regard to food and temperature the animals were divided into groups of 3 each. At intervals of several days those of each group, after preliminary starvation, were injected subcutaneously with the same preparation of insulin which was kept at a low

*Dr. H. H. Dale has particularly pointed this out and we are indebted to him and to A. Krogh for valuable advice and criticism in this work.

temperature between the periods. The same rabbit always received the same dose per kilo body weight, although the dose varied somewhat for different rabbits. The results are shown in Table VIII in which the value of **a** in the usual formula is given as the significant figure. Before drawing any general conclusions from the results it is clear that those obtained when the normal blood sugar exceeds 0.115 must be discarded on account of their being usually decidedly above the average for that animal. Thus out of a total of 63 observations, the normal blood sugar exceeded 0.115 in 12 cases and in 7 of these the value of **a** was decidedly above the average. Half of these observations were on the same day, December 26, and whether the festivities of the preceding day had affected man or beast cannot now be said. In any event these six observations must be eliminated from the series and on this account are bracketed. If we also omit the figures which are decidedly out of line (also bracketed) we find in the five animals receiving 0.1 c.c. insulin per 2 kilo body weight that the average value of **a** varied between 0.031 and

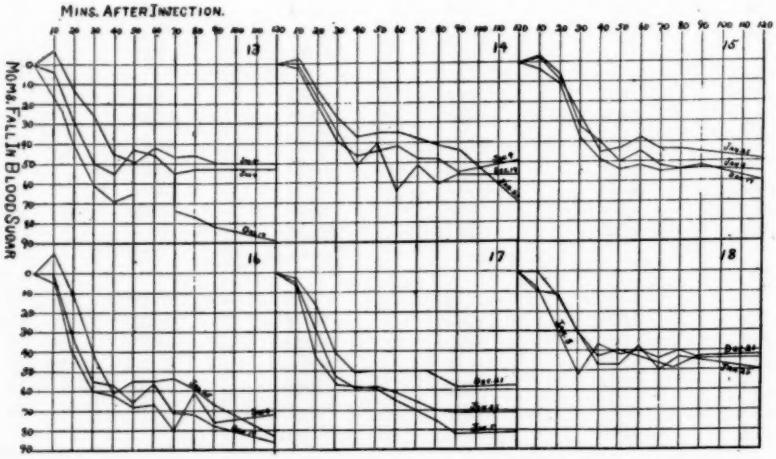


Fig. 2.—Fall in blood sugar produced by injection of the same amounts of the same preparation of insulin on 3 different occasions in 6 rabbits.
Ordinates represent mgs. fall in blood sugar.

0.049. Similarly if we take the five animals in which three or more assays were more or less satisfactory when the dose of insulin was 0.08 c.c. per 2 kg. the averages varied between 0.028 and 0.037. The grand average for the animals receiving 0.1 c.c. is 39.4 and for those receiving 0.08 c.c. it is 33. By taking an adequate number of observations with varying dosage in this manner it is possible that sufficiently reliable figures might be obtained from which curves showing the relationship between the dose of insulin and the value of **a** could be obtained and it is altogether likely that such a curve would correspond to that found by F. N. Allan for the glucose equivalents of insulin in depancreatized dogs. Since such a curve is logarithmic it is evident that the procedure followed by using the present provisional formula is fundamentally inaccurate, but until the exact form of the curve is determined it would serve no useful purpose to change the equation.

Several factors have been considered as possibly responsible for the

variations observed. These are: (1) *variability in the carbohydrate metabolism of different animals* so that the mobilization of sugar from the liver in response to hypoglycemia occurs more readily in some than in others. An animal with a low average for **a** such as Nos. 2 and 5 have presumably a more sensitive mechanism of control than those in which the average is high, such as Nos. 3 and 4.

(2) *Seasonal variations.* There is no doubt, as observed both by us and by others that there is a certain seasonable variability in the results. The assays by the usual method are, for example, almost uniformly higher in the summer months than in the winter. Although body temperature is no doubt mainly responsible for this—the higher the temperature the more susceptible the animal—there still seems to be some difference even when the temperature conditions are controlled. On this account the animals of each group were usually examined on the same days over a period of two months. Compared in this way the assays for different days in animals 2 and 3 do not show any constant seasonal parallelism although such does seem to exist when Nos. 8 and 9 are compared.

(3) *The development of an immunity towards insulin.* It is undoubtedly the case that after being used for two or three months for purposes of assay the animals become refractory towards insulin and usually they put on weight. The present observations were not continued for a sufficient time to bring out this reaction.

In another series of six rabbits, kept under strictly uniform conditions the same amounts (about one physiological unit) of the same preparation of insulin were injected subcutaneously on different occasions and the blood sugars observed at intervals of 10 minutes each for 1½ hours and then at half hour intervals. These results are shown in Fig. 2 in which the curves represent the fall in blood sugar (ordinates) at the intervals indicated (abscissae). It can be seen that not only is there considerable difference in the mean level of the curves for different animals but also that the same animal does not respond alike on different days. This divergence occurs particularly after the first 40 minutes and is dependent upon the sensitivity of the compensatory glycogenolytic mechanism. That this should vary from time to time in the same animal indicates that some unknown factor, perhaps some ductless gland other than the pancreas, exercises an influence on the sensitivity of the glycogenolytic mechanism. The slight rise which occurs during the first ten minutes in many of the observations may be due to excitement. In many of these observations the curves descend rapidly more or less in a straight line to a low level, which they reach in about forty minutes, and then either rise or become much less steep. This decided change does not usually last for long being followed either by a secondary fall or by the curve becoming horizontal. It is interesting that in a general way each rabbit responds on different occasions in much the same manner. The occurrence of the temporary rise no doubt indicates that a sudden mobilization of sugar out of the glycogen stores has occurred and its possible relationship to a hypersecretion of epinephrin has been suggested by Cannon.[8]

BIBLIOGRAPHY

[1]Banting, Best, Collip, Macleod and Noble: Amer. Jour. Physiol., 1922, lxii, 162.
[2]McCormick, Macleod, Noble and O'Brien: Jour. Physiol., 1923, lvii, 234.
[3]Fraser, D. T.: Jour. Lab. and Clin. Med., 1923, viii, No. 7.
[4]Allan, Frank N.: Amer. Jour. Physiol., 1924, lxvii, 275.
[5]Eadie, G. S. and Macleod, J. J. R.: Amer. Jour. Physiol., 1923, lxiv, 285.
[6]Macleod, J. J. R.: Jour. Metabolic Research, 1922, ii, No. 2.
[7]Collip, J. B.: Jour. Biol. Chem., 1923, lvi, 513.
[8]Cannon, W. B.: Proc. Am. Physiol. Soc., December, 1923.

INSULIN AND THE SKIN*

By Ernst F Müller, M.D., and H. B. Corbitt, A.M., New York City, N. Y.

I.

INVESTIGATIONS by one of us[1] on the biologic processes in the human body have led to the conclusion that the skin possesses special functions which manifest themselves in certain stimulative processes. It has been demonstrated that injections of nonspecific substances *within the skin, produce a different and more pronounced effect than if the same substances were administered subcutaneously.* Thus, an intradermal injection of 0.1 to 0.2 c.c. of Aolan, a nonspecific lactalbumin preparation will produce a manifest body reaction (for instance, a decided increase in urethral excretion in cases of posterior gonorrhea). Subcutaneous, intramuscular or intravenous injection of an equal quantity of the same substance is quite ineffective and only the administration of 50 to 100 times the amount used for intradermal injection will produce the same effect.

Another important finding: intradermal injection of a nonspecific substance is followed by a reflex resulting in turn in a decrease of leucocytes in the peripheric vessels; if protein solutions have been employed this reaction usually lasts from 25 to 30 minutes; after saline injections, it is only of short duration. This decrease is observed in healthy individuals as well as in those bodily ill, the leucocyte count being reduced two-thirds to one-half, this applies even to cases of severe leucocytosis, for instance, in pneumonia and in leukemia.

Further study has revealed a close relationship between the skin and the parasympathetic part of the involuntary nervous system inasmuch as the vessels in the area of the splanchnic react to intradermal stimulation which reaction in turn entails the decrease of leucocytes in the peripheric vessels. The same close relationship exists between the skin and the involuntary nervous system, so that intradermal injections influence the vessels in the areas of infection and a focal reaction ensues.

This brief outline of the research conducted along these lines sufficiently proves the close relationship between the skin and the inner organs and the ability of the skin to modify certain biologic processes of the body by way of the involuntary nervous system.

---

*This contribution is made possible through the special research fund of the H. A. Metz Laboratories, Inc., New York City.

Further study of these problems revealed that the effect not only of nonspecific but also of specific substances is considerably modified by the skin. Though the facts themselves are beyond doubt, explanation of the underlying causes of this phenomenon in specific therapy is not available.

In the course of an extensive series of investigations on the resorption of toxins by the skin, it was shown that the latter is able to detoxify various substances. A slow resorption by the skin would naturally play an important part in modifying the effect of any injected fluid. However, recent experiments with specific substances have produced results which cannot be explained even on the basis of a difference in resorption.

A report on a certain group of these tests constituting a unit in themselves is given below:

II.

Experiments were made with insulin and rabbits were used for the tests. Animals do not respond to intradermal injections with nonspecific preparations, nor is it possible to produce leucopenia in animals by means of intradermal injection. It is, therefore, not feasible to apply the results of these newer experiments directly to man.

The tests were carried out as follows: Rabbits were fasted for 18 to 20 hours and their blood sugar was determined. They were then injected with equal quantities of insulin, simultaneously, by both the subcutaneous and the intradermal route.

TABLE I

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN* UNIT PER KG.	INSULIN* TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %
44	1470	subcutaneous	0.68	1.0	0	0.120	
					2	0.0388	–67.5
					4	0.087	–27.5
					6	0.126	+ 5.0
61	1470	subcutaneous	1.0	1.4	0	0.108	
					2½	0.0417	–61.2
					4½	0.080	–26.0
					6¼	0.100	– 7.4
					24	0.127	+25.0
68	1665	subcutaneous	1.0	1.6	0	0.135	
					2	0.073	–46.0
					4	0.121	–10.4
					6	0.128	– 4.1

*Insulin U 20, Nos. 73246-741132.

Banting and Best[2] have designated as 1 unit of insulin (per kg. bodyweight) the quantity which in subcutaneous injection decreases the blood sugar of a normal rabbit from about 0.100 to 0.110 per cent to about 0.045 per cent. This has since been accepted as the physiologic unit for insulin treatment.

To insure results that would be comparable with the fundamental investigations of Banting and Best and their collaborators, preliminary tests were

made to determine the change produced in the blood sugar in our rabbits with 1 unit of insulin (Iletin), as purchased in the open market and also to determine the time required to restore the original values.

The blood-sugar determinations were made according to the method of McLean[3] as modified by Hastings and Hopping[4] each figure representing the average of two to three titrations. This method has been tried and found accurate in previous work.[5]

It is evident from these preliminary tests using the subcutaneous method of injection that ordinarily the lowest blood-sugar value (about 50 per cent of the original) is attained about two hours after the injection; four hours after injection the decrease is only about 25 per cent and after six hours, at the latest, the original figures again obtain.

Two rabbits were injected simultaneously with equal amounts of insulin taken from the same bottle, the only difference being that one rabbit was injected *subcutaneously* while the other received the injection by the *intradermal* route.

TABLE II

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
71	2480	intra-dermal	1	2.5	0	0.123		
					2	0.052	–57.7	2 wheals
					4	0.062	–49.7	
					6	0.114	– 8.1	
67	2235	subcu-taneous	1	2.2	0	0.120		
					2	0.064	–46.7	
					4	0.134	+11.7	
					6	0.124	+ 3.3	

There is a decided difference between the results of subcutaneous and of intradermal injection. In rabbit 71, the blood sugar was reduced for four hours to 50 per cent of the normal value, and six hours after the injection the original figures had not yet been regained.

Rabbit 67 was injected *subcutaneously* with an equal amount of insulin; two hours later the level obtained by the intradermal injection had not been reached (– 46.7 per cent as against – 57.7 per cent). Four hours after the injection, the original values were not only attained but were exceeded while rabbit 71 of the intradermal route still had – 49.7 per cent.

In the following experiments, a third animal was given an intradermal injection of Aolan, a nonspecific lactalbumin; the amount of solution injected being equal to that employed for insulin injection. These controls were intended to show whether nonspecific stimulation itself has any influence on blood-sugar content.

Two hours after subcutaneous insulin injection the values decreased about one-half (– 52.2 per cent) a figure which was not quite reached in the case of intradermal administration. Considering the variability in animals from a practical standpoint, the difference is negligible. After four hours the readings were similar; after subcutaneous insulin injection the blood-sugar content was only 9.4 per cent below normal while the rabbit which

had been given intradermal injections still had – 48.8 per cent, in other words, the value corresponded to those attained in the same rabbit two hours previously and greatly resembled the previous figures after two hours for case 69 (subcutaneous injection). At that period the effect of intradermal injection considerably exceeded that of subcutaneous administration. Even at the end of six hours, the intradermal injections held the blood sugar to the level of the two hour test after the subcutaneous injection. The difference in these values is, however, of little practical importance. From the second to the

TABLE III

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
69	2520	subcutaneous	1	2.5	0	0.117		
					2	0.055	–52.2	
					4	0.106	– 9.4	
					6	0.121	+ 2.5	
58	2690	intradermal	1	2.7	0	0.121		2 wheals
					2	0.065	–46.3	
					4	0.062	–48.8	
					6	0.055	–54.5	
70	2130	intradermal	Aolan	0.4	0	0.119		2 wheals
					2	0.121	0	
					4	0.124	0	
					6	0.127	0	

TABLE IV

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
64	2325	subcutaneous	1	2.3	0	0.147		High initial sugar
					2	0.072	–51.0	
					4	0.134	– 8.9	
					6	0.135	– 8.2	
67	2100	intradermal	1	2.1	0	0.112		2 wheals
					2	0.065	–42.0	
					4	0.078	–30.4	
					6	0.098	–12.5	
71	2210	intradermal	1	2.2	0	0.145		1 wheal
					2½	0.073	–49.7	
					4¼	0.080	–44.8	
					6⅓	0.116	–19.3	

sixth hour after injection, the effect is in the neighborhood of 50 per cent, a value which is retained for at least four hours, in other words, the effect is three times as great.

Blood-sugar readings showed no variations after intradermal injections of Aolan.

In a third series of tests, the rabbits of former experiments were used again but this time the animals which had formerly received subcutaneous injections were injected by the intradermal route and *vice versa.*

After two hours there was a decrease of 51.0 per cent after subcutaneous

injection as compared with a decrease of 42.0 per cent to 49.7 per cent after intradermal injections, in other words, the effect was practically the same. After four hours, however, there was a striking difference, subcutaneous: – 8.9; intradermally: – 30.4 and – 44.8. After six hours, the figures were – 8.2 per cent as compared with –12.5 per cent and – 19.3 per cent. Once more, there is a slight but distinct difference. If the difference observed after intradermal injection as between one wheal and two wheals is merely incidental or whether importance may be attached thereto, we are not prepared to say.

Further experiments made with one unit were productive of similar results. The rabbits employed for our test showed a rather high yet normal percentage of blood sugar. Subcutaneous administration of 1 unit insulin (per kg.) produced a decrease in the first tests and later also in the controls, which, however, did not reach the figure of 0.045 per cent as required by Banting and Best. A decrease of 50 per cent, however, was the rule.

In comparing the results of the subcutaneous and the intradermal method of administration, it will be found that the effect of an equal dose of insulin is considerably prolonged if given by the intradermal route. This is rather remarkable and difficult of explanation. Factors of resorption alone could not explain these variations, for practically the same effect on the blood sugar is evident with both methods within a period of two hours. If, after intradermal injection only part of the injected insulin can take effect immediately, due to slow resorption, surely the effect on the blood sugar could not be equal to that produced by the larger quantity of insulin which is resorbed immediately after subcutaneous injection. Then also the prolonged lowering effect on the blood sugar can hardly be explained in this manner.

Assuming that a subcutaneous dose is resorbed and takes effect on whatever factors regulate the blood sugar, within thirty minutes after administration, this would by no means afford an explanation for the increased effect observed after intradermal injection which is of equal intensity, yet is of two hours' longer duration. In animals the wheal disappears comparatively soon; it is never visible longer than thirty minutes. Even if it is assumed that the difference in reaction of the two methods of injection is due to the slower resorption by the intradermal route, it would be expected that the period of initial low sugar would merely be postponed. Furthermore, the delay might be expected to last a little longer than the time during which the wheal is discernible, a period within thirty minutes after the injection. This explanation becomes improbable when it is considered that the lowest value of the blood sugar for both methods is reached after one to two hours; the difference exists solely in the period during which this low value is maintained.

It might be claimed that a sudden rise in the rate of sugar metabolism in the interval between tests would escape notice by our method of illustration in curves. This is impossible for the reason that a reduction of the blood sugar to a slightly lower figure leads to convulsions, a symptom never observed in any of the animals used in our tests with the dosages used above. Thus a specific effect is materially increased by changing the mode of administration.

These facts are best illustrated by the following tests. Blood-sugar determinations were made at hourly intervals.

Even though in rabbit 74 the decrease is not so decisive, the test nevertheless reveals the interesting fact that a very low value was reached within one hour after intradermal injection, the figure being considerably lower than the blood-sugar percentage of the control. The later findings in the control are not absolutely indicative, as the blood for the sugar tests had to be taken from the heart, no blood being obtainable from the aural vein. The rabbit died of hemorrhage into the pericardium.

TABLE V

RABBIT NO.	WT.	METHOD OF INJECTION	INSULIN* UNIT PER KG.	INSULIN* TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
74	2350	intra-dermal	0.5	1.2	0	0.130		
					1	0.087	−33.1	
					2	0.090	−30.8	
					3	0.095	−34.6	
					4½	0.110	−15.4	
					6	0.125	− 3.8	
75	1950	subcu-taneous	0.5	1.0	0	0.155		Impossible to bleed from ear. All bleedings from heart. Died after 4½ hrs.
					1	0.120	−28.2	
					2	0.096	−38.1	
					3	0.088	−43.3	
					4½	0.073	−53.0	At death.

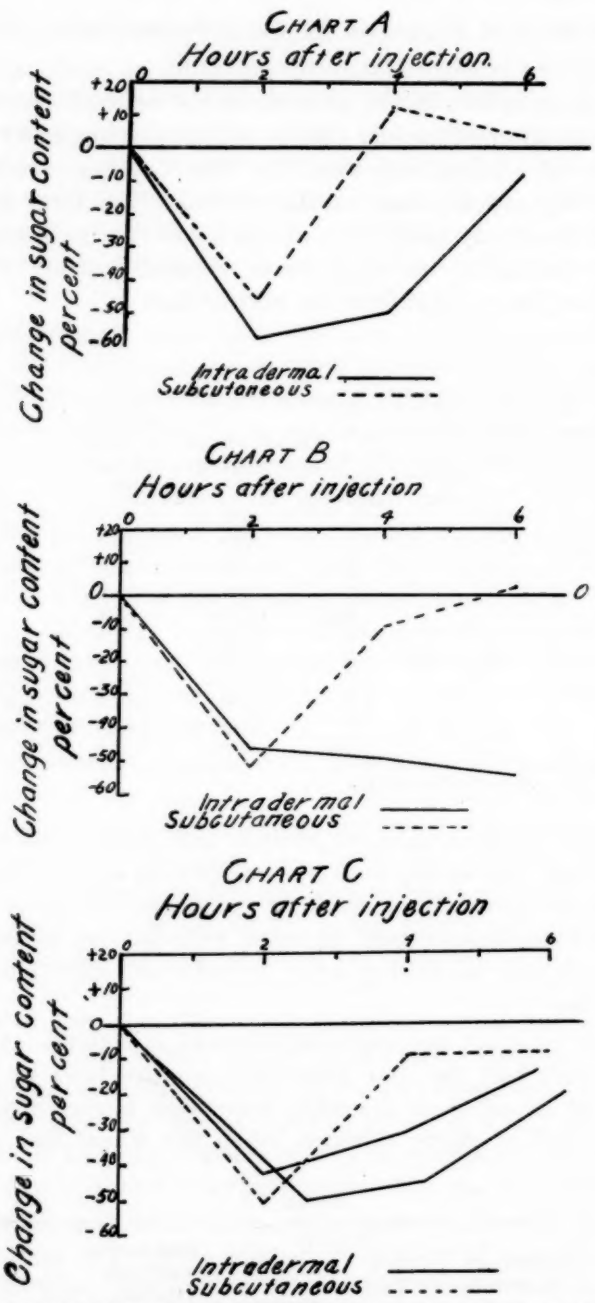
*Insulin U 10. Nos. 74049-741149.

Every effort has been made to exclude any possible source of error. It has been said that the wheals entirely disappeared after thirty minutes; not even the slightest infiltration remaining visible or evident to the touch. To insure for the subcutaneous test, an exact reproduction of the changed conditions of resorption prevailing after intradermal injection, the following tests were made:

One rabbit received the usual intradermal injections. A second animal was injected with half the dose about five minutes later on the assumption that in case of intradermal injection resorption begins after this lapse of time. At the end of thirty minutes, when the wheal had disappeared the

TABLE VI

RABBIT NO.	WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %
72	2800	subcutaneous	0.25	0.7	0	0.122	
			0.25	0.7	½	0.097	−20.5
		(in 2 doses)			2	0.089	−27.1
					4	0.098	−19.7
					6	0.117	− 4.1
73	2260	intradermal	0.5	1.13	0	0.111	
					½	0.067	−39.6
					2	0.063	−43.2
					4	0.118	+ 6.3
					6	0.108	− 2.7



Charts *A*, *B*, and *C*—Table VII.—Graphic presentation of the changes in blood-sugar content after subcutaneous and intradermal administration of 1 unit insulin (per kg.), in rabbits.

blood sugar was determined, whereupon the second rabbit received the second half of the subcutaneous dose. The results were as follows:

The tests are not entirely uniform but they do show that intradermal injection is more effective. In rabbit 73, the decrease after one-half hour is lower than in the animal treated subcutaneously and this advantage is maintained for two hours.

A graphic demonstration of a number of other tests will best illustrate the different results.

Table VII. (See charts.)

Summarizing our experiences: *There is evident after intradermal administration a prolongation of the effect of insulin on the blood sugar of normal rabbits.* We are not able to give an explanation of these facts.

Beyond doubt, the skin as an individual organ is partly responsible for this effect; also the factor of resorption plays an important part. However, it alone does not suffice to explain this phenomenon.

III.

In order to complete our experiments, the following questions remained to be answered: (1) What is the effect of small intradermal doses; and (2) What is the maximum tolerated dose and what is the lethal dose in intradermal injection?

TABLE VIII

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
72	2885	intradermal	0.5	1.4	0	0.107		Slight loss of insulin from injection.
					2	0.095	−11.2	
					4	0.097	− 9.3	
					6	0.122	+14.0	
					7¼	0.119	+11.2	
73	2445	subcutaneous	0.5	1.2	0	0.102		
					2	0.057	−44.2	
					4	0.107	+ 4.9	
					6	0.110	+ 7.8	

TABLE IX

RABBIT NO.	RABBIT WT.	METHOD OF INJECTION	INSULIN* UNIT PER KG.	INSULIN* TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %
58	2665	subcutaneous	0.5	1.33	0	0.120	
					2	0.065	−45.8
					4	0.097	−19.2
					6	0.111	− 7.5
70	2030	intradermal	0.5	1.0	0	0.113	
					2	0.05	−55.8
					4	0.042	−62.8
					6	0.080	−29.2
					7½	0.136	+20.3

*Insulin U 10. Nos. 74049-741149.

The following tests were also made with half a unit per kg. Insulin was used in a strength of 10 units per c.c., to insure full dose injections, intradermally.

Intradermal injection of one-half dose (½ unit per kg.) will also result in prolonging the effect beyond that of subcutaneous injection even though the first decrease in blood sugar does not reach the same low level. Rabbit 72 (Table VIII) received an indefinite amount as part of the solution (20 units in 1 c.c.) leaked out again.

TABLE X

RABBIT NO.	WT.	METHOD OF INJECTION	INSULIN UNIT PER KG.	INSULIN TOTAL UNITS INJECTED	TIME AFTER INJECTION (HOURS)	BLOOD-SUGAR CONTENT %	CHANGE IN SUGAR CONTENT %	REMARKS
64	2260	intradermal	2.0	4.5	0	0.166		
					2	0.05	−70.0	
					4	0.081	−51.2	
					6⅓	0.117	−29.5	
71	2170	subcutaneous	2.0	4.3	0	0.170		Mild convulsions 3 hrs. after injection.
					2	0.043	−74.7	
					4	0.070	−58.8	
					6	0.093	−45.3	
67	2015	intradermal	3.0	6.0	0	0.124		Convulsions at 1¾ hrs.
					2	0.046	−63.0	" at 2¾ hrs.
					4	0.05	−59.7	Died after 4 hrs.
69	2345	subcutaneous	1.97	4.6	0	0.124		
					1⅚	0.039	−68.6	
					2			Mild convulsion.
					2½			2 grams glucose intraperitonally.
70	1825	intradermally	3.23	5.9	0	0.127		
					1¾			Convulsions 2 grms. glucose.
					1¾			
					3			Convulsions 2 grms. glucose.
					4⅓			Convulsions 2 grms. glucose.
69	2330	intradermal	1.97	4.6	0	0.142		
					1½	0.039	−71.8	
					2½			Convulsions.
					3			Convulsions, 2 grms. glucose.
70	1910	subcutaneous	3.23	6.2	0	0.118		Convulsions, 2 grms. glucose.
					1⅓			
					3⅓			Convulsions, 2 grms. glucose.

Table X gives information as to the fatal dose.

The experiments with toxic and fatal doses supplement the earlier tests and reveal interesting additional data. Some preliminary tests were made with larger amounts of insulin in order to ascertain the effect of mode of injection upon toxicity. From Table X it is seen that possibly somewhat larger doses may be withstood when given intradermally. More work remains to be done on this phase of this subject. This table, at first sight, hardly seems to coincide with the earlier experiments. It may be, however, that the effect obtained with the aid of the skin cannot be enhanced by overdosage.

At any rate, the fatal dose for intradermal injections may be higher than the fatal dose for subcutaneous injections.

IV.

The above experiments afford an entirely new aspect of the functions of the skin and of their rôle in specific biologic processes in the body. They show that the effect of insulin on rabbits is increased when the agent is given intradermally. It is further indicated that the effect of the toxic dose may

be lowered by the intradermal route. The practical scope of insulin medication has thus been materially broadened both as to intensity and duration of effects.

As has already been said, we are not able at this time to offer an explanation of these observations. Differences in the factor of resorption alone do not suffice as an explanation. Nor does there seem to be any direct relation to observations previously made on the human skin. These latter represented the magnification of numerous, very small nonspecific stimulations by way of a reflex of the parasympathetic nervous system; the character and intensity of this stimulation could not be changed, it was a truly nonspecific effect.

Our newer experiments with insulin show an increase of a specific effect on the one hand, and on the other indicate a detoxication of the fatal dose without a corresponding prolongation of the effect. Thus the practical results of these tests attribute to the skin as an individual organ an active rôle distinctly different from any heretofore recognized properties. In this active rôle, the skin participates in various ways in a specific process. Therefore, this characteristic of the skin as a reflex action which reaches the vessels by way of the involuntary nervous system cannot be classified, but must assume various closely related processes, each contributing its share. The skin itself cannot influence sugar metabolism; this has been proved by our tests with nonspecific controls. This regulation of sugar metabolism in cases of overdosage can be attained only in the presence of the specific ferment.

The above are the facts developed from our observation in the animal. At this writing, it is impracticable to construct far-reaching synthesis on the basis of these practical findings, because the relation between the skin and the blood-sugar changes following intradermal injections of insulin is not known. An explanation is to be offered when further observations have been made and when our present series of studies along these lines have been completed. At present, investigations are being made along these lines with other substances including Suprarenin. The results will be available for publication in the near future. Nevertheless, it is believed that the above findings are of sufficient interest to warrant a report at this time as they reveal that the skin as an individual organ plays an important part also in *specific* biologic processes.

BIBLIOGRAPHY

[1]Müller, E. F.: München med. Wchnschr., 1921, lxviii, No. 29; 1922, lxix, No. 43, No. 51; Zeitschr. f. d. ges. Exper. Med., 1923, xxxii, 120.
[2]Banting, F. G. and Best, C. H.: Am. Jour. of Physiol., 1922, lxvi, 162.
[3]McLean: Biochem. Jour., 1919, xvi, 135.
[4]Hastings, A. B. and Hopping, A.: Proc. Soc. Exper. Biol. and Med., Feb., 1923, xx, 254.
[5]Dubin, H. E. and Corbitt: Proc. Soc. Exper. Biol. and Med., 1923, xxi, 16.

THE EFFECT OF INTRAVENOUS ADMINISTRATION OF SODIUM CITRATE IN TWO CASES OF HEMOPHILIA*

By Lee D. Cady, M.D., and E. Lee Shrader, M.D., St. Louis, Mo.

THE use of sodium citrate administered intravenously was first reported by Weil[1] in 1915. He noted that instead of lengthening the coagulation time of the blood as it did *in vitro,* there was a prompt shortening by one-half the control coagulation time. The next year Ottenberg[2] administered 0.6 gm. Intravenously to a hemophiliac with marked drop in the coagulation time, followed by a prolongation of the coagulation time as noted forty-eight hours later. Kinsella and Broun[3] gave 1.0 gm. doses to influenza patients who had a hemorrhagic tendency. These patients invariably showed a prompt shortening of coagulation time which lasted at least twenty-four hours. No study was made of its effects on the platelets. These patients, however, usually had a markedly lowered platelet count. In 1921 Neuhof and Hirshfeld[4] reported on the slow intravenous administration of doses as large as 8 gm. on patients without blood diseases. By repetition of the injection after twenty-four hours they believed that they were able to keep the coagulation time down. There was no evidence of intravascular coagulation, thrombus or embolism. As previously noted by Kinsella and Broun[3] too rapid administration caused prompt respiratory embarrassment, tingling and twitching of the lips, etc. The following year Neuhof and Hirshfeld[5] again reported on the routine use of sodium citrate injections in surgical patients as a means of preventing or stopping hemorrhage. They used 9 gm. for intramuscular injection and 6 gm. for intravenous injection in adults. Some of their patients did not show any reduction of coagulation time following sodium citrate injections. Except in cases of purpura and hemophilia no harmful effect on the blood was observed. However, sustained shortening of coagulation time could not be maintained longer than four or five days by repeated injections. "Large doses" were given to two hemophilic patients with slight primary drop in the coagulation time followed by a marked prolongation far beyond the original time. In three cases of purpura there was an immediate and continued prolongation of the coagulation time. They concluded that blood disease where there is platelet deficiency, is a clear contraindication for the use of sodium citrate injections. They are supported in this belief by Rosenthal and Baehr[6] who believe the paradoxical shortening of blood coagulation after intravenous administration of sodium citrate is due to its injurious effects on the circulating blood platelets, thereby liberating their thromboplastic substance (cytozyme) into the plasma. They did not find any changes in the calcium, fibrinogen or antithrombin. Within

*From the Medical Clinic, Barnes Hospital, Department of Internal Medicine, W. U., School of Medicine.
Received for publication, August 10, 1923.

ten to fifteen minutes after the injection the blood platelets were reduced the maximum amount and returned to normal in about thirty minutes. Where there is a numerical deficiency in platelets, as in ducks, and in patients with purpura and hemophilia, no beneficial effects, but actually undesirable results were obtained. Therefore, they concluded that, "in hemorrhagic blood diseases * * *, its use is strictly contraindicated."

The present report is based on the study of the effects of sodium citrate injections in two cases of hemophilia. In the first case (Table I) the sodium citrate was injected intravenously in 100 c.c. of physiologic saline solution. Platelet counts were made on capillary blood by the Wright and Kinnicutt method.[7] Clotting time determinations were made on venous blood drawn through a 20-gauge needle and placed in chemically cleaned, flat-bottomed 10 x 40 mm. glass tubes. The average time when the tubes could be inverted without displacement of the blood was taken as the end point.

TABLE I

DATES	SODIUM CITRATE	TIME AFTER INJECT.	CLOTTING TIME	BLOOD CA. MGM. 100 C.C. BLOOD	REMARKS
7/18/22	4 gm.	0	60 min.		Given preparatory to extraction of tooth.
		30 min.	23 min.		
		10 hrs.	41 min.		Practically no bleeding from gum.
7/19/22					No bleeding from gum. Clot moderately firm.
7/20/22 8:30 a. m.	4 gm	0	43 min.	9.6	Given prior to extraction of three teeth.
		30 min.	76.5 min.	9.4	
		3 hrs.	55 min.	9.6	
5:00 p. m.					Considerable oozing from gums. Clots very soft, gelatinous, easily dislodged, not retractile.
5:30 p. m.	4 gm.				Given because of continued oozing; clot soft, jelly-like.
7/21/22 2 p. m.					500,000,000 typhoid vaccine subcutaneously.
8:00 p. m.					Reaction from vaccine with less bleeding.
7/22/22					Bleeding practically stopped.
					Local application of $FeCl_2$. Thromboplastin and Adrenalin.
8/1/22	6 gm.	0	108 min.		One tooth extracted. Bleeding slightly at times for four days.
		4 hr. 20 min.	65 min.		8/2/22. Acute pyelitis with fever. Local applications of Thromboplastin and Adrenalin.

In the second place (Table II) 4 gm. of sodium citrate in a 28 per cent solution in distilled water were injected intravenously. Platelet counts were made by the Kristenson method[8] on capillary blood. Clotting time determinations were made as above described.

The second injection (Table III) of sodium citrate was 3.3 gm. in 25

TABLE II

TIME	CLOTTING TIME MINUTES	PLATELET COUNT	FRAGILITY	DIFFERENTIAL %				
				S. M. N.	L. M. N. TRANS.	P. M. N.		
						N.	E.	B.
Control	89		.37–.21	26.4	15.8	56.6	0	1
10 min.		107,000						
35 min.	109	89,000		20.9	21.9	56.8		
55 min.	90	92,800	.37–.21	31.4	11.3	57.1	.3	0
1 hr. 35 min.	196	103,200		30.6	14.4	54.4	0	0
2 hr. 40 min.	150	79,200	.37–.25	20.3	23.3	55.4	0	0
4 hr. 55 min.	30	124,000		21.6	10.5	67.1	.8	.3
29 hrs.	12	160,000					.3	.6
6 days	7							
8 days	31							
9 days	54							
11 days	24							
13 days	32							
	etc.							

per cent distilled water. Platelet counts were made by the Kristenson method[8] on venous blood taken from the same specimens used for clotting time determinations. We find that this method is more satisfactory in several respects than the Wright and Kinnicutt method.[7] Differential counts of 300 to 400 cells were made at the same time the platelet counts were made. The blood was drawn through 20-gauge needles coated with a thin layer of ether-paraffin into 10 c.c. glass syringes similarly prepared. The blood was placed in 10 x 70 mm. round-bottomed glass tubes previously coated with a thin layer of ether-paraffin and the clotting times were taken as the average time when the tubes could be inverted without displacing the blood.

TABLE III

TIME	CLOTTING TIME	PLATELET COUNTS	WHITE BLOOD CELLS	DIFFERENTIAL %				
				S. M. N.	L. M. N. TRANS.	P. M. N.		
						N.	E.	B.
Control	22 hrs. 25 min.	415,000	6,600	18	5.7	75.5	2.5	.25
Control	23 " 55 "	335,000						.0
30 min.	23 " 26 "	290,000	9,800	24.2	6.2	68.0	.97	
60 "	20 " 32 "	290,000	9,500	14	6.1	75.5	2.13	.3
90 "	23 " 52 "	375,000	8,400	22.6	4.4	72.0	.53	.26
160 "	15 " 48 "	380,000	6,600	23.2	13.2	60.5	1.82	1.1
24 "	4 " 31 "	227,000	6,050					
48 "			9,000	22.6	4.6	72.4	.38	
72 "	1 " 8 "	228,000	6,700					
120 "	4 " 12 "		8,200					

CASE M-9702. Age fifty-one, white, male, jeweler.

Two brothers died in infancy, cause unknown. One brother died of hemorrhage following a slight injury. Patient has no male grandchildren.

Slight cuts or abrasions always produce excessive bleeding. Since the age of six years, the patient has had frequent attacks of hematuria, melena, (without other gastro-intestinal symptoms), questionable retinal hemorrhages and bleeding from gums. The hematuria occurred from two to six times a year and lasted from two to seven days. Cystoscopy subsequent to an attack of hematuria and prior to admission resulted in a diagnosis of bladder tumor.

Physical examination revealed a secondary anemia and numerous foci of infection; namely ethmoidal-sphenoidal sinusitis, severe pyorrhea and pyelitis. Diagnosis of pulmonary

tuberculosis was made from physical signs, x-ray and sputum examinations. The heart was normal. Blood pressure 95/45. Many blood clots were found on cystoscopy, but no evidence of tumor. Red blood cells, 3,200,000; white blood cells, 7,600; hemoglobin 70 per cent. Blood smears normal. Wassermann negative. Platelet count varied between 132,000 and 156,000.

Sodium citrate was given to prevent bleeding from gums following extraction of teeth. Three injections (1, 3 and 4) were given just prior to extraction. The second injection of citrate was given after extraction, during bleeding from gums (Table I).

CASE M-10484. Age thirty-four, white, male, clerk.

The maternal grandfather, two cousins by a maternal aunt, and two nephews by two different sisters are likewise hemophiliacs. Patient's condition was diagnosed at the age of three, and at various times since then has had severe hemorrhages. Since the age of twenty has had chronic tonsillitis, chronic infectious arthritis, and has a mitral insufficiency. There is a long history of conditions such as hemarthroses, hematomata, hematuria, epistaxis, etc. Admitted to Barnes Hospital during an acute exacerbation of tonsillitis and arthritis of the left knee.

Laboratory data: Red blood cells, 4,380,000; white blood cells, 8,200; hemoglobin 80 per cent. Blood smears normal; bleeding time 90 seconds; blood Wassermann negative.

Four grams of sodium citrate were injected intravenously (Table II) and about twenty-four hours later the left knee was aspirated under local anesthesia. About 1 c.c. of very bloody fluid was obtained, which clotted within the syringe in less than one minute. No hemorrhage about puncture wound occurred. The patient had an uneventful hospital course and was discharged. One week after discharge from hospital had a large ecchymosis on left arm. Later he began having more extensive ecchymoses on arms and left thigh. The teeth were cleaned by a dentist and gums bled three or four days. Hemorrhages became worse and patient reentered hospital in bad condition.

Physical examination revealed nothing new except marked anemia and large, tense and painful ecchymoses in the left thigh and left upper arm. Red blood cells, 2,950,000; white blood cells, 10,950; hemoglobin 75 per cent. Blood smears were normal except for an occasional red blood cell showing basophilic degeneration. The bleeding time was 50 seconds. The hemorrhagic areas steadily increased and the anemia became worse. Two days after admission 3.3 gm. of sodium citrate in a 25 per cent solution were administered intravenously (Table III). The red blood cell count was 3,096,000.

DISCUSSION

As noticed by previous authors these patients had certain definite symptoms if the sodium citrate was injected too rapidly. The only symptoms allowed to occur were subjective. Extreme care was taken not to push the administration of the drug to the point where objective signs were likely to occur. The first thing noticed by the patients was a sour or saline taste in the mouth. No effort was made to avoid the occurrence of this symptom, but when the patients began to have tingling or quivering sensations in the lips, or any sense of respiratory embarrassment whatever, the administration was discontinued until these sensations were gone. The citrate solution was more dilute in the first case and was given by the gravity method, while in the second case the citrate solution was concentrated and given with a syringe. In either case the reactions were the same if the solution was given too rapidly. In order to eliminate any effect of dilution, concentrated sodium citrate solution was given to the second patient.

There are certain accidents incident to the use of the concentrated solution which may occur. While administrating it in the second case, a small amount was accidentally injected into the subcutaneous tissues and infil-

trated the radial nerve. The patient had immediate and excruciating pain at the site of infiltration, rapidly followed by paresthesia and total paralysis throughout the radial distribution. These symptoms gradually passed off, and after an hour no trace remained. No injury at the site of infiltration remained except a slight tenderness for one day.

While the above observations can only be considered as fragmentary and were not made by the same methods, they are essentially parallel in results. Intravenous injections of sodium citrate cannot always be expected to cause immediate shortening of clotting time. It may even cause an increase in the clotting time for a few hours, but this is followed by a decrease in the clotting time. This temporary increase in the clotting time is of relatively little importance considering the definitely prolonged original clotting time. Neither of the patients can be said to have been symptomatically worse during or following this temporary prolongation of clotting time. On the other hand, it cannot be said that the hemorrhages of the hemophilic patients will be controlled by intravenous sodium citrate injections alone. Sometimes, however, considerable benefit may occur as shown in Table III where the control clotting time was 22 hours and 25 minutes and there was a more or less steady but slow shortening over three days to one hour and eight minutes. Repeated injections do not seem to have the beneficial effects of single injections. The platelets were diminished immediately after the injections and slowly returned to normal. On the other hand, the white blood cells were concurrently increased followed by a return to normal 2 to 3 hours later. No significant changes occurred in the differential counts.

CONCLUSIONS

1. There is usually some prolongation of clotting time immediately after sodium citrate injection, ultimately followed by a marked shortening of the clotting time.

2. These effects on clotting time seem to be associated with changes in the blood platelets.

3. Intravenous administration of sodium citrate of hemophilic patients appears to be harmless and not contraindicated when not repeated too frequently.

BIBLIOGRAPHY

[1]Weil, R.: Sodium Citrate in the Transfusion of Blood, Jour. Am. Med. Assn., 1915, lxiv, 425.

[2]Ottenberg, R.: The Effect of Sodium Citrate on Blood Coagulation in Hemophilia, Proc. Soc. Exp. Med. and Biol., 1916, xiii, 104.

[3]Kinsella, R. A. and Broun, G. O.: Changes in the Blood in Influenza, Jour. Am. Med. Assn., 1920, lxxiv, 1070.

[4]Neuhof, H. and Hirshfeld, S.: Slow Intravenous Administration of Large Doses of Sodium Citrate, New York Med. Jour., 1921, cxiii, 95.

[5]Neuhof, H. and Hirshfeld, S.: The Intramuscular Administration of Sodium Citrate. A New Method for the Control of Bleeding, Ann. Surg., 1922, lxxvi, 1.

[6]Rosenthal, N. and Baehr, G.: Paradoxical Shortening of Blood Coagulation After Intravenous Administration of Sodium Citrate, Pro. Soc. Exp. Biol. and Med., 1922, xx, 130.

[7]Wright, J. H. and Kinnicutt, R.: A New Method of Counting the Blood Platelets for Clinical Purposes, Jour. Am. Med. Assn., 1911, lvi, 1457.

[8]Kristenson, A.: A New Method for the Direct Counting of the So-called Blood Platelet in Man, Acta Medica Scandinavica, 1922, lvii, 301.

THE FOUR-HOUR URINE TEST

A Comparison Between the Twenty-Four Hour and the Four-Hour Specimens in Sixty Normal Individuals*

By Henry M. Feinblatt, M.D., Brooklyn, N. Y.

STUDY upon a four-hour urine test was begun in the hope that this method might yield reliable quantitative readings and at the same time measure the concentrating power of the kidneys. If it can be shown that this is the case, this test will find a definite place among laboratory methods, first as an improvement upon the routine examination of a chance morning specimen, which has no value other than in a qualitative sense, and secondly as a substitute for the twenty-four-hour specimen under conditions in which that method cannot be conveniently employed.

The interpretation of a quantitative urine analysis cannot be made without a knowledge of the volume of urine and the conditions of food intake for the period during which the specimen is collected. Inferences drawn from the specific gravity must likewise be made in the light of these factors.

The standard metabolism period has been fixed by custom at twenty-four hours. This period should be begun immediately after passing the night urine, thus discarding waste products secreted the night before.

There is no room to doubt the reliability of deductions based upon a twenty-four-hour specimen of urine correctly obtained. But it is a matter of common knowledge that mistakes are frequently made in the collection of such samples. The patient absent-mindedly discards a voiding; or an orderly adds the urine to the wrong bottle. Furthermore, it is often difficult to secure rigid adherence to the dietetic program during the metabolic period.

The choice of a twenty-four-hour period for the collection of a specimen of urine for quantitative analysis was, in the first place, quite arbitrary. It has never been shown that a shorter period will not satisfactorily serve this purpose.

Recently, Folin[1] published a report of the quantitative examination of 20 three-hour morning specimens of urine. It was this investigation which suggested the advisability of instituting a comparison between the twenty-four-hour sample and that obtained over a shorter metabolic period. A four-hour period was chosen because of the convenience of collecting the specimen between 8 o'clock in the morning and noon and because 4 is a divisor of 24. This latter arithmetic relation offers a simple means of computation in estimating twenty-four-hour from four-hour findings.

*From the Department of Internal Medicine, Long Island College Hospital, Brooklyn, N. Y.

Received for publication, Aug. 20, 1923.

THE SCOPE OF THIS INVESTIGATION

The object of this study was to open up an avenue of research as to the comparative amounts of the various normal constituents of the urine contained in the twenty-four-hour and the four-hour specimens respectively. This comparison, if carried out in sufficient detail, should show just how much reliance can be placed in quantitative determinations on specimens collected over an abbreviated metabolic period.

The first items that suggest themselves in such a comparative study are the specific gravity and the quantitative values for chlorides and for urea. The question of the urea output has been left for a later investigation. The present report is based upon comparative observations on the specific gravity and the chloride content in the twenty-four-hour and the four-hour samples.

HOW THE DATA WERE OBTAINED

The present investigation was conducted upon sixty apparently healthy medical students. At 8 o'clock in the morning the urine was voided and discarded. Immediately thereafter, a standard meal consisting of two slices of bread with butter and one glass of milk was ingested. No water was allowed before 12 o'clock. The urine passed during this period together with the noon voiding constituted the four-hour specimen.

After noon the subjects were permitted to resume their habitual routine, with the exception that all of the urine was collected up to and including the voiding at 8 o'clock the following morning. Thus the twenty-four-hour period was inclusive of the four-hour period studied.

The tests conducted upon these samples concerned the volume, the specific gravity and the total amount of chlorides. The latter were estimated by Volhard's method. In the four-hour specimens, the total chlorides for the twenty-four-hour period were computed by multiplying the reading obtained by six. This procedure was done on the assumption, subsequently justified by the parallelism observed, that the rate of elimination of chlorides is fairly constant.

ANALYSIS OF THE DATA

The degree of parallelism existing between the twenty-four-hour and the four-hour specimens is indicated by Table I as regards the specific gravity, and by Table II as regards the chloride content.

TABLE I

PARALLELISM OF TWENTY-FOUR HOUR AND FOUR-HOUR SPECIMENS AS REGARDS SPECIFIC GRAVITY

Average specific gravity of 24-hour specimens		1022.57
Average specific gravity of 4-hour specimens		1021.27
Difference of 2 points or less	32 specimens	
Difference of 2 to 4 points	18 specimens	
Difference of 4 to 6 points	6 specimens	
Difference of 6 to 8 points	2 specimens	
Difference of 8 to 10 points	2 specimens	

The average specific gravity of the twenty-four-hour specimens was 1022.57; that of the four-hour specimens was 1021.27. In 32, or 53 per cent, of the comparisons, the two specimens agreed within two points; in 50, or 83 per cent, the variation did not exceed four points.

TABLE II

PARALLELISM OF TWENTY-FOUR HOUR AND FOUR-HOUR SPECIMENS AS REGARDS CHLORIDE CONTENT

Average chloride content of 24-hour specimens	14.19 gm.
Average chloride content of 4-hour specimens	14.66 gm.
Difference of not more than 2 gm.	27 specimens
Difference of 2 to 4 gm.	22 specimens
Difference of 4 to 6 gm.	5 specimens
Difference of 6 to 8 gm.	0 specimens
Difference of 8 to 10 gm.	4 specimens
Difference of 10 to 12 gm.	2 specimens

Note: In the four-hour specimens, the total chloride content was estimated by multiplying the reading obtained from the sample by 6.

The average chloride content of the twenty-four-hour specimens was 14.19 gm.; that estimated from the four-hour specimens was 14.66 gm. In twenty-seven, or 45 per cent, of the comparisons, the difference between the two specimens did not exceed 2 gm. of chlorides; in 49, or 82 per cent, the variation did not exceed 4 gm.

CLINICAL USE OF TEST

The degree of parallelism here observed between the twenty-four-hour and the four-hour specimens in regard to their specific gravity and chloride content is sufficiently close to suggest that the four-hour urine test may prove to be of considerable value for clinical purposes. Further work must, of course, be done, particularly in reference to the urea content.

Study of the urinary output over a long metabolic period is open to the decided objection that it is irksome to the patient and that one can never be sure of full cooperation. In this respect, the Ambard maximum concentration test is impracticable, because it imposes too great a hardship upon the subject.

For clinical purposes, I have since modified the breakfast to consist of two eggs, an average portion (100 gm.) of farina gruel, one slice (30 gm.) of baker's white bread with a helping (20 gm.) of neufchatel cheese, and one glass (200 gm.) of milk. This furnishes a meal containing about 28 gm. of protein, 38 gm. of carbohydrate, 26 gm. of fat, and 1 gm. of sodium chloride. For seasoning, 1 gm. of sodium chloride may be allowed in addition to that already contained in the food. The fuel value of this meal is approximately 525 calories.

SUMMARY

Study upon a four-hour urine test was begun with the idea that this method might meet two indications: (1) that it might replace the routine examination of a single chance specimen, which has no value other than one qualitative, and (2) that it might serve as a substitute for the twenty-

four-hour examination under conditions in which that method cannot be conveniently employed.

The present investigation was conducted upon sixty apparently healthy medical students, in whom comparative studies as to specific gravity and chloride content were made upon the urine eliminated in the twenty-four-hour and the four-hour metabolic periods respectively.

It was found that the specific gravity readings in the twenty-four-hour and the four-hour samples were pretty much in accord. In more than half of the comparisons, the specific gravity of the two specimens agreed within two points; in 83 per cent, the variation did not exceed four points. The average specific gravity of the twenty-four-hour specimens was 1022.57; that of the four-hour specimens was 1021.27.

A similar degree of parallelism was found to obtain in the relation between the chloride output in the twenty-four-hour specimen and that estimated from the quantitative reading in the four-hour sample. In 45 per cent of the comparisons, the difference between the two specimens did not exceed 2 gm. of chloride; in 82 per cent, the variation was not greater than 4 gm. The average chloride content of the twenty-four-hour specimens was 14.19 gm.; that estimated from the four-hour specimens (by multiplying the quantity in the four-hour sample by 6) was 14.66 gm.

Further comparative quantitative analyses should be made with the other normal urinary ingredients, particularly urea. Although the scope of this work is too limited, in that parallel studies were made only in respect to the specific gravity and the chloride content, the results here obtained would indicate that the four-hour urine test is of value in meeting the conditions which suggested its employment.

REFERENCE

[1]Folin, O.: Laboratory Manual of Biological Chemistry, 1922, p. 121.

THE RELATIONSHIP OF BLOOD-SUGAR CONTENT TO KIDNEY PERMEABILITY AND GLYCOSURIA*

By Henry J. John, Cleveland Clinic

THE assumption that the point of kidney permeability for sugar is determined by the level of the blood-sugar content at the moment that sugar first appears in the urine may in certain cases seem not to be in accord with the actual observations.

For example, when in a given patient (1) glycosuria is found when the blood sugar is at a certain level and (2) on the following morning no sugar is found in the urine at a lower blood-sugar level, we conclude that the point of kidney permeability for sugar lies between these two blood-sugar

*Received for publication, August 28, 1923.

concentrations and by a series of subsequent readings this point of permeability can be quite definitely located. Subsequent observations, however, may indicate what appears to be a puzzling discrepancy for sometimes (3) sugar may be found in the urine when the blood sugar is at an even lower level than in (2); or there may be no sugar in the urine when the blood-sugar level is higher than in (2) but below (1).

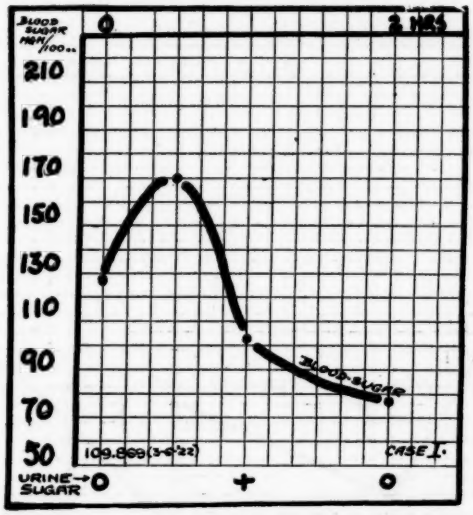


Chart I.

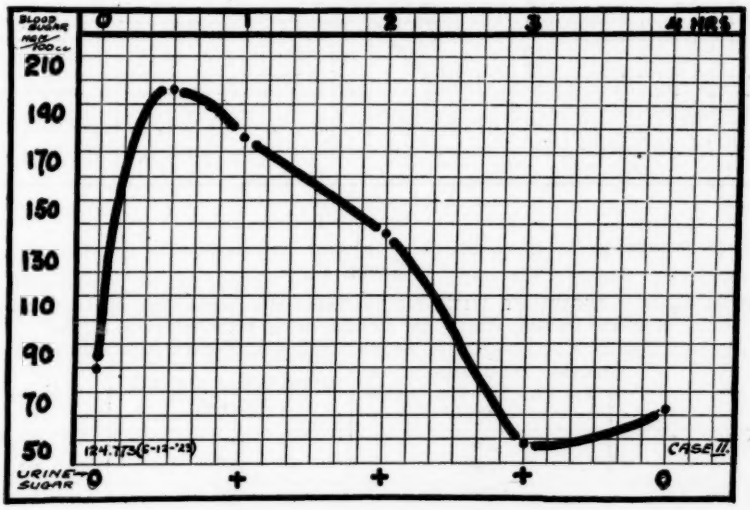


Chart II.

At first it seems difficult to correlate these apparent discrepancies, especially in the absence of abundant data for study and comparison.

It is very essential that the cause of such variations in the relation of the blood sugar to the presence or absence of sugar in the urine be understood, especially in view of the present intensive studies of the effects of insulin, in which some investigators are interpreting their results on the

basis of blood-sugar content, of the sugar content of the urine, or of both.

For this reason I am offering data from two cases selected from a number in which similar observations were made.

In Case I Chart I shows that when the blood-sugar content was 126 mg. per 100 c.c. there was no sugar in the urine, one hour later there was sugar in the urine although the blood-sugar level had fallen to 102 mg. per 100 c.c. These observations seem illogical until we take into consideration the period

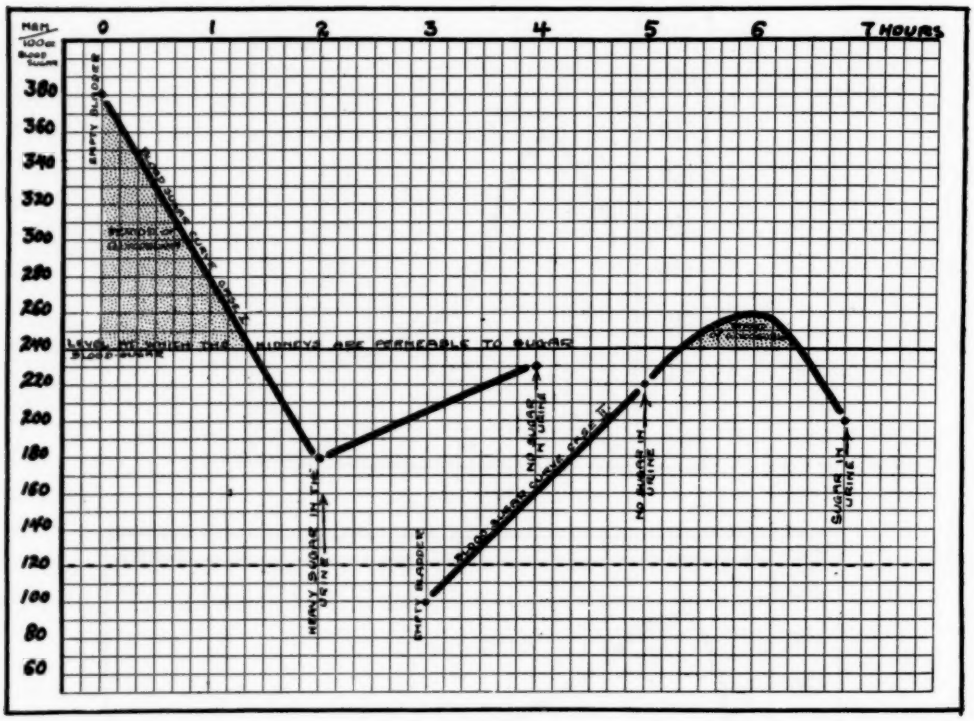


Chart III.

TABLE I

APPARENT LACK OF CONFORMITY WITH THE ESTABLISHED RELATION OF GLYCOSURIA TO THE KIDNEY PERMEABILITY TO SUGAR IN A HYPOTHETICAL CASE

	RENAL PERMEABILITY AT MG. PER 100 C.C. BLOOD SUGAR	BLOOD SUGAR MG. PER 100 C.C.	URINE SUGAR
Case No. I	240	180	2 plus
	240	230	negative
Case No. II	240	220	negative
	240	200	plus

preceding the second observation during which there was a hyperglycemia reaching beyond the point of the renal permeability. The sugar in the urine at the second reading therefore is readily explained.

In Case II, as is shown by Chart II, the point of renal permeability as determined by comparing the first and third readings must lie somewhere between 90 and 146 mg. per 100 c.c. Nevertheless when the blood sugar is

58 mg. per 100 c.c. as in the fourth reading there is sugar in the urine. As in Case I this is explained by the hyperglycemia of the preceding period.

To illustrate further the necessity of interpreting the presence or absence of glycosuria in its relation to the blood-sugar content of the preceding period during which the urine was secreted, I am offering the accompanying charts of two hypothetical cases, the assumed data in which is in accord with our clinical observations on various cases similar to those cited above.

As is shown by Table I and Chart III two blood-sugar determinations and synchronous urine-sugar determinations are given for each case. It is supposed that the point of renal permeability is the same for each case, namely at a blood-sugar concentration of 240 mg. per 100 c.c. In the first case when the blood sugar is 180 mg. per 100 c.c. we find a heavy sugar content of the urine; but when the blood-sugar concentration is 230 mg. per 100 c.c. we find no sugar in the urine. This discrepancy seems incomprehensible until we note as indicated by the chart that the first determination of the urinary sugar was made just after the period during which the blood sugar was above the level of kidney permeability, while the second measurement of the urinary sugar was taken more than two hours later. These facts offer a sufficient explanation of the presence of sugar in the urine at the first determination even though the blood sugar is below the point of kidney permeability.

In the second case there is no glycosuria when the blood sugar is 220 mg. per 100 c.c. but two hours later when the blood sugar is only 200 mg. per 100 c.c. there is glycosuria. In this case at the time of each reading the blood sugar is below the point of permeability, but as the curve shows, there has been an intervening period during which, perhaps after a meal, the blood sugar has been above the level of kidney permeability. The second reading taken immediately after that period of postprandial hyperglycemia shows a residue of sugar in the urine.

CONCLUSION

The glycosuria must be interpreted not only in its relation to the synchronous blood-sugar content, and to the level of kidney permeability to sugar, but also in its relation to the blood-sugar variations during the preceding period during which the urine was secreted.

MITOCHONDRIA CONTENT OF THE THYROID AS AN INDEX OF THE ACTIVITY OF THE GLAND*

By William Bolt, M.D., Winnipeg, Canada

THE question of the relation of cytoplasmic inclusions to cellular activity is one that has received considerable attention during the last few years. Heretofore the nucleus has been considered the alpha and omega of cell life and the cytoplasm merely a vehicle or a feeder for it. However, as a result of investigations by Gatenby,[1] Cowdry,[2] and others the question has arisen as to whether after all the cytoplasm has not been wrongly ignored, and whether its contents do not play a very important part in determining the fate of the cell.

Prominent among the protoplasmic (or living inclusions) of the cell, as opposed to the deutoplasmic (or dead inclusions) are the mitochondria, which, according to Gatenby, are numerous granules or filaments scattered throughout the cytoplasm, and staining red with acid fuchsin after fixation with potassium bichromate or osmic acid. They also may be demonstrated by other more complicated methods, e.g., Benda's, Kapsch Altman's.

In 1916 Goetsch[3] published an account of his investigations into the mitochondria of the thyroid, but so far no effort seems to have been made to confirm his findings. Attention was drawn to this piece of work by the case of a patient in the Winnipeg General Hospital with a thyroid that caused toxic symptoms but which both macroscopically and microscopically seemed to be simply a colloid goiter. Accordingly it was thought worth while to examine a number of thyroids for mitochondria, the methods used and the results obtained forming the basis of this article.

The method employed was practically the same as Cowdry's modification of the Altman-Bensley stain, the technic being as follows:

A. *Fixation:*

1. Thin strips of fresh thyroid, not over 1 mm. thick, were placed in Regaud's mixture (3 per cent potassium bichromate 80 per cent, commercial formalin neutralized with magnesium carbonate 20 per cent) for four days, the fluid being changed daily.

2. The next eight days the tissue was kept in 3 per cent potassium bichromate, the solution being renewed every second day.

3. Tissue was dehydrated, cleaned and embedded in wax, and sections about 4 μ thick were cut, affixed to albuminized slides and cleared by passing through xylol and alcohol to water.

*From the Department of Pathology, Winnipeg General Hospital.
Received for publication Aug. 28, 1923.

B. *Staining:*

1. The slides were immersed in 1 per cent potassium permanganate for 30 to 60 seconds, then rinsed in 5 per cent oxalic acid for 30 seconds.

2. After being thoroughly washed in distilled water, they were stained for six minutes at 60° C. in Altman's anilin acid fuchsin (10 c.c. acid fuchsin in 100 c.c. anilin oil).

3. Next they were cooled, dipped in distilled water, and differentiated by staining for an instant in 1 per cent aqueous methyl green solution.

4. Then they were drained, dehydrated quickly in absolute alcohol, cleared and mounted in Canada balsam in the ordinary way.

The resulting picture showed the mitochondria as bright red granules or filaments on a green background.

To gain an idea of the normal number of mitochondria in the gland, the thyroids from three necropsies performed within two hours of death were examined. These showed practically the same picture as the normal parts of glands removed at operation. In one other necropsy case the mitochondria were found to be quite appreciably increased. But this was in the case of a child of thirteen months who died of gastroenteritis. No further opportunity presented itself of checking this finding to see if the thyroids of young children are richer in these granules than the glands of adults.

Turning to operative cases, a study was made of a number of cases of *exophthalmic goiter.* In these the mitochondria were found to be largely and uniformly increased, the cytoplasm being infiltrated by a dense mass of small red granules. In one specimen, however, they were much less numerous, and in some areas hardly increased at all. This was in the case of a patient who had had a goiter since childhood but in whom the symptoms had subsided markedly and the basal metabolism had fallen from +80 to +40 under two weeks' rest in bed.

The *adenomata* also showed a marked increase in mitochondria, but not uniformly so, as in the exophthalmic cases, the increase being more marked in some areas so as to give the section a mottled appearance. In one series of ten cases nine showed this patchy arrangement of the granules. The tenth case showed an appreciable general increase similar to the picture found in the exophthalmic sections and it is noteworthy that this specimen was one in which the slide made from a piece of tissue cut from the center of the adenoma very much resembled the picture of Graves' disease in that there was marked hyperplasia with "budding" into the lumina of the acini, and the lining cells were in more than one layer in places. Another case, in which there was very little increase in the mitochondria, resembled the exophthalmic case mentioned above, in that the patient had improved markedly under rest, and the basal metabolism had fallen from plus 70 to plus 40.

The *colloid goiters* however were the most interesting as it was really to observe the mitochondrial content of these that this work was undertaken. Of all the thyroids examined six colloid goiters seem worthy of some individual attention.

1. The first was from a patient with no toxic symptoms whatever. Ordi-

nary histologic section showed a typical colloid goiter. The section stained for mitrochondria showed no increase, their number being about the same as in a normal thyroid. This case was used as a standard for the other colloid goiters.

2. The second patient had had a toxic adolescent goiter with moderate toxicity, e.g., tachycardia and some heart irregularity, for eight months. There was bilateral enlargement of the gland. Unfortunately there was no determination of the basal metabolic rate in this patient. Following the operation, the symptoms subsided and the patient has been in good health since.

Sections showed a colloid goiter with distinct hyperplasia but, although a number of slides were examined, no increase in mitrochondria could be detected. No explanation could be found to account for this finding which was directly at variance with the cases following. It is possible there was some undetected error in technic, such as leaving the gland too long before placing it in the fixing fluid. The one point in which this case differs clinically from the rest, however, is that it was an adolescent goiter, whereas the other cases studied were those where the goiter was of longer duration and in older patients.

3. The third patient had had swelling of the neck for eight years with symptoms the last two, nervous spells, insomnia, weakness, fatigue and loss of weight being the predominating features. On examination the gland was markedly enlarged, especially the right lobe, and there was marked tremor of the fingers. The basal metabolic rate, however, was only plus 23. Microscopic section showed a colloid goiter with evidence of hyperplasia in some areas, but in addition throughout the entire section there was a decided increase in the number of mitochondria, very apparent in the typical colloid areas both in the lining cells and in the interstitial tissues.

4. The fourth case resembles the third in that it was an old colloid causing moderate toxemia with a basal metabolic rate of plus 32. Microscopic section showed colloid goiter with some hyperplasia and general increase in mitochondria, though not as marked as in the previous case.

5. The fifth patient had had a goiter for seven years with symptoms of fatigue, dyspnea, nervousness and tremor the past year. Basal metabolic rate was plus 35. Following operation the symptoms disappeared and patient has been in good health since.

Microscopic section showed colloid goiter with slight evidence of hyperplasia and general increase of mitochondria throughout the whole section.

6. The last case was that of a patient who had had an enlarged thyroid for three years, symptoms being exaggerated by pregnancy two years before. Patient showed nervousness, dyspnea, exophthalmos, bilateral enlargement of the gland and had a basal metabolic rate of plus 35. Following operation the symptoms disappeared and patient's health has been quite good since. The eyes are much less prominent though they still protrude somewhat.

Microscopic section showed a typical colloid goiter with large acini lined by low epithelium and distended with colloid. In this case however, no evi-

dence of hyperplasia could be found anywhere. Yet the mitochondria were markedly increased and quite profuse throughout the sections.

It will be seen that the findings in these cases correspond with the results obtained by Goetsch. In the adenomata and the exophthalmic goiters the increase in mitochondria goes hand in hand with other changes which are revealed by ordinary staining methods so that the examination for mitochondria is not so helpful in these cases. In the case of colloid goiters with toxic symptoms however, the examination for mitochondria may reveal changes otherwise undetected. For although in most of the cases there was some hyperplasia, yet the amount in some cases was very small and in one case no sign of it could be detected, yet in all the cases examined the mitochondria were markedly and generally increased. As these were all cases of enlarged thyroids with recent development of toxic symptoms one is inclined to wonder if the effect of the stimulus that causes the change is not first shown on the cytoplasm particularly the mitochondria, followed later by further changes, e.g., hyperplasia.

I am indebted to Dr. William Boyd and Miss M. Van Romberg of the Department of Pathology for advice and assistance in the preparation of the material for this article.

REFERENCES

[1]Gatenby, J. B.: Cytoplasmic Inclusions of Germ Cells, Quar. Jour. Micros. Sci., Jan. 1920. The Modern Technique of Cytology, Quar. Jour. Micros. Sci., March, 1920.
[2]Cowdry, E. V.: Functional Significance of Mitochondria, Am. Jour. Anat., May, 1916.
[3]Goetsch, E.: Functional Significance of Mitochondria in Toxic Thyroid Adenomata, Johns Hopkins Bulletin, May, 1916, xxvii, 129.

LABORATORY METHODS

COMPARISON OF THE WASSERMANN, SACHS-GEORGI AND KAHN TESTS FOR SYPHILIS*

By Elizabeth W. Rockstraw, and Michael J. Bent, M.D.,† New York City

THE early work of Porges and Meier and others which demonstrated that many syphilitic serums gave visible precipitates with various lipoid solutions failed to develop reliable methods for the diagnosis of this disease. The more recent modifications of these reactions by Meinicke and Sachs-Georgi have given results which closely parallel those of the Wassermann test. These tests have the advantage of simplicity but most of those who have compared them with the Wassermann reaction have agreed that they were slightly but definitely less sensitive.

Still more recently Kahn[1] has developed through a series of modifications a method which differs principally in using larger amounts of serum and more concentrated antigen solutions, thus making the precipitation more rapid and more easily visible, and the test more delicate. The results which he has obtained by this method parallel very closely those with the Wassermann test. It seems that a careful comparison of a series of serums tested in parallel by the method of Sachs-Georgi, by that of Kahn and by the Wassermann test would be of value.

The following report covers 1022 such comparative tests made during the past year.

TECHNIC

The Wassermann tests were carried out in one-tenth the volume recommended by Wassermann and Citron, using 0.02 and 0.01 of patients' serum in the tests, 0.02 in the serum control and proportionate amounts of the other reagents making the total volume about 0.5 c.c. The tests were performed in triplicate using one cholesterinized antigen with water-bath fixation for one hour, and two simple alcoholic extracts of beef heart with ice-box fixation for four hours. Both were finished by adding sensitized cells and incubating for 15 minutes, the time limit for the titration of complement.

The Sachs-Georgi tests were run with three antigens, Rice's[2] technic being followed. No serum controls were used, however, as both Parker and Haigh[3] and later Rice found that only antigen controls were necessary.

*From the Department of Bacteriology, Columbia University.
†Fellow in Medicine of the National Research Council.
Received for publication, December 3, 1923.

In the Kahn precipitations we followed in all the essential details the procedure described in his paper of September 1922, and because of the large amount of serum required we were able to use only one cholesterinized and one alcoholic antigen with a serum and antigen control.

THE ANTIGEN

Fresh beef heart was freed from fat, blood vessels and endocardium, passed several times through the meat grinder, spread on glass plates and dried with an electric fan. When dry, it was again ground several times with the meat grinder, the result being a fine powder, part of which was extracted with ether at room temperature, and part with a Sohxlet apparatus. Both proved to be equally good, but of course the extraction at room temperature consumed more time. When the supernatant ether was colorless, the heart was spread on filter paper to dry. Alcohol was added in the proportion of 20 grams of powdered heart to 100 c.c. alcohol, extraction taking place in the ice-box for nine days, and one day at room temperature. The extract was then filtered and a portion set aside for use as plain alcoholic antigen. To another portion we added 4 mg. cholesterin per 100 c.c.

THE SERUM

All specimens were tested within five days of the time the blood was withdrawn and were inactivated as for the Wassermann reaction. Some stood for some hours after inactivating before the antigen was added, and to others the antigen was added immediately after inactivation. No nonspecific or doubtful results were observed with these latter as reported by Münster[4] with the Sachs-Georgi tests. Cloudy serums and those containing hemoglobin gave satisfactory results, although the cloudy fluids required more careful observation.

SALINE

A solution of 0.85 per cent sodium chloride in distilled water was used for diluting the antigen. We also used 0.9 per cent sodium chloride as for the Wassermann test and diluted the antigen with distilled water instead of the salt solution with very little, if any, difference in the precipitation, but these last were not included in our 1022 cases, and only a few (24) tests were run.

DILUTION OF ANTIGEN

The antigen was diluted by measuring the alcoholic solution into one large test tube, the saline into another of the same size. The saline was then poured into the antigen with moderate speed and the mixture poured back and forth several times. The same size test tube was used and the same method was carried out each day. In every instance we diluted our antigen freshly for the day's work. In preliminary tests with each alcoholic extract we prepared mixtures with varying proportions of saline: 1-1, 1-2, 1-3, and 1-4. We found that our cholesterinized antigen gave best results

when diluted with three parts, and the alcoholic with two parts of salt solution. When we used less saline there was a precipitate with the antigen control and with Wassermann-negative sera and with some clinically non-syphilitic.

SERUM CONTROL

With each serum we also ran a serum control, 0.3 c.c. of serum and 0.05 c.c. of alcohol and saline, the proportion in which the antigen was diluted with saline. In no instance did we observe precipitation in the control, and we believe that these might be omitted in the future.

ANTIGEN CONTROL

One antigen control was used for each antigen dilution, 0.05 c.c. plus 0.3 c.c. saline.

THE TEST AS SET UP

Tube 1. Alcoholic antigen test: 0.3 serum plus 0.05 antigen dilution (1-2).

Tube 2. Cholesterinized antigen test: 0.3 serum plus 0.05 antigen dilution (1-3).

Tube 3. (Serum control) 0.3 serum plus 0.05 alcohol diluted with saline (1-2).

Tube 4. Antigen control: (Alcoholic antigen control) 0.3 salt solution plus 0.05 antigen dilution (1-2).

Tube 5. (Cholesterin antigen control) 0.3 salt solution plus 0.05 antigen dilution (1-3).

Each tube was shaken immediately after the addition of antigen, or alcohol and salt in the case of the serum control, rather than waiting until each tube in the rack was filled and then shaken, as the test seemed to be more sensitive when the serum and antigen were mixed immediately.

INCUBATION

The tests were incubated for eighteen hours at 37.5° C. They were then read as follows:

One large or several large clumps was called	++++
Large flocculent precipitate was called	+++
Smaller flocculent precipitate was called	++
A fine precipitate which does not dissolve on shaking	+
No change	negative

TABLE I

GROSS RESULTS

	POSITIVE	NEGATIVE	ANTICOMPLEMENTARY
Wassermann (Cholesterin)	340	682	0
Wassermann (Alcoholic)	316	704	2
Kahn	325	697	0
Sachs-Georgi	292	730	0

TABLE II

GROSS COMPARISON

AGREEMENT							
Wassermann (Cholesterin antigen)	Positive	340	Kahn	Positive	313	96.2%	
	Negative	682	"	Negative	670		
			Sachs-Georgi	Positive	267	90.4%	
			" "	Negative	657		
Simple alcoholic antigen	Positive	316	Kahn	Positive	276	92.2%	
	Negative	704	"	Negative	665		
			Sachs-Georgi	Positive	249	89.5%	
			" "	Negative	664		
DISAGREEMENT							TOTAL
Wassermann (c)	Positive		Kahn	Negative	27	39	
"	Negative		"	Positive	12		
Wassermann	Positive		Sachs-Georgi	Negative	73	98	
"	Negative		" "	Positive	25		

DETAILED COMPARISON

			Kahn					Sachs-Georgi				
Wassermann (c)			++++	+++	++	+	−	++++	+++	++	+	−
	203	++++	160	26	7	6	4	72	77	38	7	9
	70	+++	3	23	17	19	8	1	9	24	14	22
	12	++		1	4	4	3		0	4	0	8
	55	+		4	8	31	12		2	7	12	34
	682	−			2	10	670		1	9	15	657
Wassermann (a)	185	++++	136	23	9	6	11	65	61	35	10	14
	69	+++	15	19	12	8	15	6	13	17	7	26
	20	++	1	4	6	6	3		1	7	5	7
	42	+	3	9	9	6	15	1	5	10	6	20
	704	−		5	8	25	666	1	3	16	19	665
Anticomplementary	2		1			+		1				1

TABLE III

GROSS COMPARISON OF TREATED CASES

AGREEMENT							
Wassermann (c)	Positive	132	Kahn	Positive	113	86.5%	
"	Negative	99	"	Negative	87		
			Sachs-Georgi	Positive	89	75.7%	
			" "	Negative	86		
DISAGREEMENT							TOTAL
Wassermann	Positive		Kahn	Negative	19	31	
"	Negative		"	Positive	12		
"	Positive		Sachs-Georgi	Negative	43	56	
"	Negative		" "	Positive	13		

DETAILED COMPARISON OF TREATED CASES

Wassermann		Kahn					Sachs-Georgi				
		++++	+++	++	+	−	++++	+++	++	+	−
54	++++	37	10	2	1	4	13	22	13	2	4
40	+++	2	19	12	2	5	0	9	10	11	10
4	++	0	3	6	15	10	0	0	3	0	1
34	+	0	1	2	1	0	0	1	5	0	28
99	−	0	1	1	10	87	0	1	4	8	86

TABLE IV

GROSS COMPARISON OF UNTREATED CASES

Wassermann (c)	Positive	208	Kahn	Positive	200	98.9%
"	Negative	583	"	Negative	583	
			Sachs-Georgi	Positive	176	94.4%
			" "	Negative	571	

DISAGREEMENT

Wassermann	Positive	Kahn	Negative	8
"	Negative	"	Positive	0
"	Positive	Sachs-Georgi	Negative	30
"	Negative	" "	Positive	12

DETAILED COMPARISON OF UNTREATED CASES

Wassermann (c)		Kahn					Sachs-Georgi				
		++++	+++	++	+	−	++++	+++	++	+	−
149	++++	123	16	5	5	0	59	55	25	5	5
30	+++	1	8	7	13	1	1	0	14	3	12
8	++	0	0	3	3	2		0	1	0	7
21	+	0	1	2	13	5		1	2	12	6
583	−					583			5	7	571

HISTORY OF UNTREATED CASES IN WHICH THERE WAS DISAGREEMENT

Kahn Negative, Wassermann (cholesterin) Positive

Wassermann +++ — One case: Unable to get history.

Wassermann ++ — Two cases: One gave a definite history; lesion of fifteen days' duration. Enlarged inguinal glands on both sides. The other gave a history of syphilis and was diagnosed as tertiary lues.

Wassermann + — Five cases: With one there was no history of syphilis, but a dime sized lesion on posterior vaginal wall, probably traumatic; duration unknown. The other four giving a + had definite luetic histories.

Sachs-Georgi Negative, Wassermann Positive

Wassermann ++++ — Five cases: No history in one case. The others were diagnosed as syphilis and gave definite history of the disease.

Wassermann +++ — Twelve cases: No history of one case; another denies history of syphilis. The others gave definite histories of syphilis.

Wassermann ++ — Seven cases: Five gave definite histories of syphilis. One diagnosed as fibroid tumor denied history of syphilis. Another gave no history of syphilis and the symptoms did not suggest that diagnosis.

Wassermann + — Six cases: Three gave history of lues. Three denied history of lues and their diagnoses were laceration of pelvic floor, enlarged thyroid, posterior displacement of corpus uteri.

Sachs-Georgi Positive, Wassermann Negative

Sachs-Georgi + or ++ — Twelve cases: Only one gave a history of syphilis, two denied it, four are being treated for other troubles in Sloane Hospital, four in the neurologic department and three in the general clinic.

DISCUSSION OF RESULTS

The results of these reactions are shown in some detail in the accompanying tables.

We found that the Kahn test with cholesterinized antigen was more sensitive than the Sachs-Georgi, or the Wassermann with plain alcoholic antigen, but slightly less sensitive than the Wassermann with cholesterinized antigen. (Table I) The Kahn reactions with unfortified alcoholic extract were positive only with a relatively small number of the strongest reacting sera and seemed of little practical value.

It is of interest to compare in detail the Kahn and Wassermann tests with the same type of antigen because the precipitation and complement-

fixation tests are doubtless but two methods of detecting the same reaction between serum and lipoid solution. Considering first the 791 untreated cases, (Table IV) it is seen that in 730 instances the Kahn and Wassermann readings with cholesterinized extract were identical in degree (+, ++, +++, ++++) a rather remarkable agreement considering that the readings were made without direct comparison. In none of this group did we obtain a positive Kahn if the Wassermann reaction was negative. The Wassermann reaction was positive in 8 where the Kahn was negative. In only 3 of these, however, was the Wassermann stronger than "+," a reaction of slight diagnostic value. It is interesting, nevertheless, that of these 8 cases 6 were considered syphilitic by clinicians. It would be premature on the basis of present experience to suggest replacing the Wassermann with colesterinated antigen by the Kahn test, but as far as our experience goes very few diagnoses would be missed by such a substitution.

Turning to the group of treated cases (Table III) we find somewhat more frequent and marked disagreement between the two methods. The most interesting point observed was that in a number of cases the Wassermann reaction became negative while the Kahn persisted, a factor which might be of importance in deciding on cessation of treatment.

If we compare the Wassermann with plain alcoholic antigen with the Kahn still more discordant results appear. While the percentage of positive tests in these Wassermanns was smaller, this method gave several strongly positive reactions where the Kahn was negative.

The Sachs-Georgi test was less sensitive than the Kahn and more frequently disagreed with the Wassermann reaction. Of the untreated cases a small, but by no means negligable number (17) gave negative reactions although the Wassermann was strongly positive, and of 12 cases which gave a weak positive Sachs-Georgi and a negative Wassermann, only one showed clinical evidence of syphilis. We did not detect any such nonspecific reactions in our Kahn series.

The Kahn test with spinal fluids seemed of no value. Of 24 examined, 19 gave positive Wassermanns and 5 were negative. The Kahn test gave only 4 strong positives, 3 weak positives and the remainder (17) were negative.

The modified technic of the Kahn test,[11] with which we have had only brief experience, appears much more delicate. Treated cases especially show stronger positive reactions than with the Wassermann and in several instances the Kahn has given a weak positive where the Wassermann was negative. We hope later to report on a series of tests done with the new technic.

PREVIOUS REPORTS

Other workers have recorded similar findings in regard to the Kahn test.

Russell D. Herrold[5] used saturated ammonium sulphate to throw down the syphilitic "reagin," redissolved it in a small amount of normal salt solution and performed a ring test, using a modified Kahn antigen also. He

reports a large number of positives with blood serums as well as spinal fluids in which the Wassermann was repeatedly negative.

A. K. Detweiler[6] compared a series of 2000 serums using a cholesterinized antigen and reports an agreement of 94.2 per cent, the greatest discrepancy being with treated cases and spinal fluids. It is interesting to note that 6 pneumonia cases in his series gave positive Wassermanns during the height of the fever, but later when the fever subsided the Wassermann was negative while the Kahn remained negative throughout.

W. B. Moody[7] reports an agreement of 98 per cent with a ring test using a Kahn antigen modified by Herrold. He does not state the Wassermann method by which he made his comparison.

Max M. Strumia[8] reports an agreement of only 83.4 per cent, using a Kahn cholesterinized antigen and 82.7 per cent with a Kahn noncholesterinized antigen. The comparison was made with the Kolmer[9] method of complement-fixation.

Janet C. Holmes[10] compares 1000 cases using practically the same method of complement-fixation that we have employed. She finds the Kahn more sensitive in treated cases and reports 248 positive Wassermanns and 294 positive Kahns.

SUMMARY

1. The Kahn precipitation test agreed with the Wassermann reaction with cholesterinized antigen in 96.2 per cent of 1022 cases. The percentage agreement with untreated cases was 98.9 per cent; with treated cases 86.5 per cent.

2. It is in general more sensitive than the Wassermann with simple alcoholic antigen, but failed to detect some strong positive reactions obtained by this method.

3. It is more sensitive than the Sachs-Georgi reaction and more frequently agrees with the Wassermann than does the latter.

4. The Kahn test gives a useful control for the Wassermann because it is simple to perform, easy to read and relatively free from danger of technical errors.

5. It will probably be valuable in controlling therapy because it sometimes detects a persistent abnormality in serums of treated cases that have become Wassermann-negative.

REFERENCES

[1]Kahn: Arch. Dermat. and Syphil., 1922, v, 570. Ibid, 1922, v, 734. Ibid, 1922, vi, 332.
[2]Rice: Jour. Infect. Dis., 1922, xxxi, 444.
[3]Parker, F. and Haigh, A.: Arch. Dermat. and Syphil., 1921, iv, 67.
[4]Münster: Jour. Am. Med. Assn., 1917, p. 944, extract.
[5]Herrold, R. D.: Journal Am. Med. Assn., 1923, lxxxi, 203.
[6]Detweiler, H. K.: Jour. Am. Med. Assn., 1923, lxxxi, 815.
[7]Moody, W. B.: Jour. Am. Med. Assn., 1923, lxxx, 383.
[8]Strumia, M. M.: Arch. Dermat. and Syphil., 1923, viii, 50.
[9]Kolmer, J. A.: Am. Jour. Med. Sci., 1923, clxv, No. 3.
[10]Holmes, J. C.: Jour. Am. Med. Assn., 1923, lxxxi, 294.
[11]Kahn: Jour. Am. Med. Assn., 1923, lxxxi, 88.

A MODIFICATION OF THE SACHS-GEORGI TEST, USING ACTIVE SERUM*

By S. D. Joffick, M.D., Peking, China

IT has been shown that a portion, and sometimes all, of the substances in syphilitic blood which are responsible for a positive Wassermann reaction may be destroyed when the serum is subjected to heat at 56° C. (Seelman[1]).

Hugo Hecht[2] states that "by inactivation some of the fixing substances of the syphilitic serum are lost." Traube[3] found "a change in the physical state of the sera which causes a lowering of their dispersion rate." Liebermann[4] found an increase in the alkalinity of the inactivated sera, sometimes to a marked degree. Lecomte du Nouy[5] says that "the surface tension of pure sera heated at 55° C. decreases progressively and regularly."

A change in the serum by inactivation which results in a partial or complete destruction of its fixing power in the Wassermann test may influence the precipitating power of the same serum in the Sachs-Georgi test for syphilis.

If we explain complement-fixation in the Wassermann reaction as a result of an invisible precipitation of hydrophile colloids with absorption of complement, it follows that by removing the "fixing substance" from the serum there may also be a decrease, or a complete elimination, of the precipitate in the Sachs-Georgi test. On the other hand, the presence of complement in a fresh positive serum (active) prevents flocculation.

P. Neukirch[6] found that if he applied the Sachs-Georgi test to an active positive serum a negative result was obtained. The same was found to be true by me. As an explanation of this, Neukirch holds that complement has the power to dissolve flocculi.

In 1909 Merkl[7] found that complement was destroyed by 5 per cent salt solution or stronger. Later Meinicke and other workers performed their tests with active serum, using a 2 per cent saline solution. Therefore, last year, when carrying out experiments on the mechanism of the Sachs-Georgi test,[8] I used active serum with 2 per cent saline solution as a medium, and obtained stronger but specific results. This is shown by the statistics which follow. After these experiments had been performed and the consequent conclusion drawn, a paper appeared by F. Georgi and H. Lebenstein,[9] on the influence of the saline-medium concentration on the serologic results of the Sachs-Georgi and Meinicke flocculation tests. These authors found that by the use of 1.5 per cent saline as a medium in the former test active sera were deprived of their inhibiting action on flocculation and therefore might be used in the test. They obtained, with 1.5 per cent saline as medium, the

*From the Clinical Laboratory, Department of Medicine, Peking Union Medical College, Peking, China.

Received for publication, December 28, 1923.

TABLE I

NUMBER OF SERA TESTED, 1169

With Wassermann, Sachs-Georgi Inactive and Active Methods, Meinicke and Bruck
Identical Results Obtained in 322 Positives and 658 Negatives, or 83.83%
Divergent Results in 189 Sera, as Follows:

	74 TREATED CASES					51 UNTREATED CASES					42 CLINICALLY DOUBTFUL					UNTREATED CASES CLINICALLY DIAGNOSED AS PRIMARY, 14					AS SECONDARY, 8				
Test	++++	+++	++	+ ± −	%	++++	+++	++	+ ± −	%	++++	+++	++	+ ± −	%	++++	+++	++	+ ± −	%	++++	+++	++	+ ± −	%
Wassermann	41	4	5	24	60.8 67.6	18	10	5	18	55. 64.7	16	5	11	10	50. 76.2	6		1	7	50. 42.85	6		2		100. 75.
Sachs-Georgi Active sera	49	10	8	7	80. 91.	25	11	14	1	76. 98.4	19		10	13	45.24 69.	7	4	1	2	85.7 78.87	6		2		100. 75.
Sachs-Georgi Inactive sera	10	13	14	37	31. 50.	2		7	42	4. 17.6	7	2	10	23	21.45 45.24			5	9	35.71	4		2	2	75. 50.
Meinicke	25	7	3	39	43.24 47.3	12	8	3	28	40. 45.98	13		8	21	31. 50.	3	2	6	3	78.57 35.71	5	1	1	1	87.5 75.
Bruck	27	13	5	29	54.54 60.8	24	10	4	13	66.66 74.59	16	3		23	45.24	4	1	2	7	50. 35.71	6		1	1	87.5 75.

In the divergent results belongs one case of tuberculosis with the Wassermann No. 2827. Wassermann with 3 antigens ++++, +, +. Other tests gave negative results. The patient gave a history of a primary sore two years ago.

TABLE II

Cases (146) diagnosed clinically as syphilis in which there was a divergence between the results obtained by the different tests used.

Table shows a comparison of each test with the others, i. e., the first (1) tabulates those cases in which there was a divergence between the Wassermann test (which showed ++++ or +++) and the others, which gave a ++ and + or a ± and −. If ++ and + are taken to indicate "no syphilis" there was a 4.11 per cent divergence between the Wassermann ++++ and +++ and the Sachs-Georgi test using active serum. If ++ and + are taken to indicate "syphilis" there was a divergence of 2.55 per cent.

DIAGNOSIS	WASSERMANN ++++ OR +++						SACHS-GEORGI WITH ACTIVE SERUM ++++ OR +++						SACHS-GEORGI WITH INACTIVE SERUM ++++ OR +++						MEINICKE ++++ OR +++						BRUCK ++++ OR +++					
	Sachs-Georgi																													
	Active		Inactive		Meinicke	Bruck	Wassermann		Sachs-Georgi inactive		Meinicke	Bruck	Wassermann		Sachs-Georgi active		Meinicke	Bruck	Wassermann		Inactive		Active		Bruck	Wassermann		Active		Inactive		Meinicke
Treated Syphilis		1	1	14	15	16	3	7	3	29	21	23		4		2	8	12		3				8	1	5	2	6		4		14		8
Untreated	3	2		13	8	6	5	13		22	1	13	1	6	1				3	3	6			10		2	1	10	7	1		20	2	6
Primary			2	1	1			3	1	4		2		2			1		2				2				2			1	1		
Secondary				1						2		1																					1
Total per cent	4.11	2.55	21.77	20.	16.33	15.00	21.75	15.75	41.78	37.67	25.03	24.34	21.93	21.24	3.42	2.73	1.37	6.16	5.47	8.2	7.53	4.1	4.1	13.7	12.32	5.48	4.79	14.38	12.32	8.2	3.4	24.64	23.95	11.64	10.35

same results as with inactive serum. My method of using 2 per cent saline has given considerably better results.

To find the specificity and strength of the Sachs-Georgi test with active serum 1169 sera were tested parallel with the Wassermann, original Sachs-Georgi, Meinicke and Bruck[10] methods. The Wassermann test was the original one, using one-fifth quantities. A cholesterinized beef heart extract was used as antigen. The Meinicke test was performed with two extracts, one the original horse extract of Meinicke, the other an acetone extract (my modification). Both were carefully titrated by methods giving satisfactory results.

The technic of the test remained unchanged, except for the practical suggestions given by me in another paper[11] and the use of 0.9 c.c. of a 2 per cent saline solution instead of physiologic saline when making the test. In order not to be influenced by the other tests the results were read by two different workers. The original Sachs-Georgi and Wassermann tests were not done by me. Each test was read without any knowledge of the results of the other tests.

Some of the luetic sera sent to the laboratory were marked "untreated," without any explanation as to the stage of the disease. It was therefore necessary to place these cases in a special column in the table.

That the present test is specific is shown by the percentage of the identical results obtained (83.83 per cent) in both clinically positive and clinically negative cases. The strength of the test is shown (1) by its holding longer a positive reaction in treated cases, and (2) by its percentage of positive results in every stage of the disease, as well as in the "untreated" cases.

Table I shows the response of each test to 74 cases treated, 51 untreated, 42 clinically doubtful, 14 diagnosed as primary and 8 diagnosed as secondary syphilis.

In all of these cases the test with active serum gave better results, i. e., in treated cases 80 per cent, if we consider as a positive result only reactions showing ++++ and + ++: and 91 per cent if ++ and + are also taken as indicating positive results (Table I.)

Table II shows the divergence between the results obtained by the different tests used in 146 cases clinically diagnosed as syphilis. The tests stood as follows:

1. *Wassermann (original) was positive* while	
Sachs-Georgi with active serum was negative in	4.11 to 2.55%
(Present writer's modification)	
Sachs-Georgi with inactive serum was negative in	21.77 to 20%
Meinicke was negative in	16.33%
Bruck was negative in	15%
2. *Sachs-Georgi with active serum was positive* while	
Wassermann was negative in	21.75 to 15.75%
Sachs-Georgi with inactive serum was negative in	41.78 to 37.67%
Meinicke was negative in	25.03 to 24.34%
Bruck was negative in	21.93 to 21.24%

TABLE III

SUMMARY OF RESULTS

STAGE	CLINICALLY SYPHILITIC CASES FROM THE IDENTICAL POSITIVES AND NEGATIVES														CLINICALLY AND SEROLOGICALLY NEGATIVE WITH ALL TESTS	CLINICALLY AND SEROLOGICALLY POSITIVE OR DOUBTFUL	CLINICALLY POSITIVE SEROLOGICALLY VARIOUS RESULTS	CLINICALLY DOUBTFUL SEROLOGICALLY VARIOUS RESULTS
	TREATED		(UNTREATED)		PRIMARY		SECONDARY		TERTIARY		LATENT SYPHILIS		PARASYPHILIS					
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.				
Number of sera tested showing identical results	105	109	21	9	28	27	96	7	49	7	19	9	4	2	487	681	639	42
Percentage of positives in each stage	See Table IV								89.27		68.		66.6			1169 cases		

Pos. = Positive serologic result.
Neg. = Negative serologic result.

TABLE IV

PERCENTAGE OF POSITIVES IN EACH STAGE OF THE DISEASE WITH EACH TEST

TESTS	WASSERMANN							
	Treated		(Untreated)		Primary		Secondary	
Number of sera tested								
Showing Identical results	Pos. 105	Neg. 109	Pos. 21	Neg. 9	Pos. 28	Neg. 27	Pos. 96	Neg. 7
Divergent Results — Strong positive: ++++ or +++	45	29	28	23	6	8	6	2
Divergent Results — Strong and weak positive ++++, +++, ++, +	50	24	32	18	7	7	8	
Making a Total of — Identical results plus strong positives	150	138	49	32	34	35	102	9
Making a Total of — Identical results plus strong and weak positive	155	133	54	27	35	34	104	7
Percentage of strong positives ++++ or +++	52.08		60.5		49.27		91.9	
Percentage of strong and weak Positive ++++, +++, ++, +	53.82		66.6		50.72		93.7	

TESTS	SACHS-GEORGI WITH ACTIVE SERUM								INACTIVE SERUM							
	Treated		(Untreated)		Primary		Secondary		Treated		(Untreated)		Primary		Secondary	
Number of sera tested																
Showing Identical results	Pos. 105	Neg. 109	Pos. 21	Neg. 9	Pos. 28	Neg. 27	Pos. 96	Neg. 7	Pos. 109	Neg. 105	Pos. 21	Neg. 9	Pos. 28	Neg. 27	Pos. 96	Neg. 7
Divergent Results — Strong positive: ++++ or +++	59	15	36	15	11	3	6	2	23	57	2	49		14	4	4
Divergent Results — Strong and weak positive ++++, +++, ++, +	67	7	50	1	12	2	8		37	37	9	42	5	9	6	2
Making a Total of — Identical results plus strong positives	164	124	57	24	39	30	102	9	128	160	23	58	28	41	100	11
Making a Total of — Identical results plus strong and weak positive	172	116	71	10	40	29	104	7	142	146	30	51	33	36	102	9
Percentage of strong positives ++++ or +++	57.		73.73		56.52		91.7		44.44		28.4		40.58		90.1	
Percentage of strong and weak Positive ++++, +++, ++, +	60.		87.65		58.		83.7		49.3		37.37		47.82		91.9	

TESTS	MEINICKE								BRUCK							
	Treated		(Untreated)		Primary		Secondary		Treated		(Untreated)		Primary		Secondary	
Number of sera tested																
Showing Identical results	Pos. 105	Neg. 109	Pos. 21	Neg. 9	Pos. 28	Neg. 27	Pos. 96	Neg. 7	Pos. 105	Neg. 109	Pos. 21	Neg. 9	Pos. 28	Neg. 27	Pos. 96	Neg. 7
Divergent Results — Strong positive: ++++ or +++	32	42	20	31	5	9	6	2	40	34	34	17	5	9	6	2
Divergent Results — Strong and weak positive ++++, +++, ++, +	35	39	23	28	11	3	7	1	45	29	38	13	7	7	7	1
Making a Total of — Identical results plus strong positives	137	157	41	40	33	36	102	9	145	143	55	26	33	36	102	9
Making a Total of — Identical results plus strong and weak positive	140	148	44	37	39	30	103	8	150	138	59	22	35	34	103	8
Percentage of strong positives ++++ or +++	47.57		50.62		47.82		91.9		50.		68.		47.82		91.9	
Percentage of strong and weak Positive ++++, +++, ++, +	48.68		54.32		56.52		92.8		52.08		71.6		50.72		92.8	

3. *Sachs-Georgi with inactive serum (original) was positive* while

Wassermann was negative in	3.42 to 2.72%
Sachs-Georgi with active serum was negative in	1.37%
Meinicke was negative in	6.16 to 5.47%
Bruck was negative in	8.2%

4. *Meinicke was positive* while

Wassermann was negative in	7.53 to 4.1 %
Sachs-Georgi with active serum was negative in	4.1%
Sachs-Georgi with inactive serum was negative in	13.7 to 12.32%
Bruck was negative in	5.48 to 4.79%

5. *Bruck was positive* while

Wassermann was negative in	14.38 to 12.32%
Sachs-Georgi with active serum was negative in	8.2 to 3%
Sachs-Georgi with inactive serum was negative in	24.64 to 23.95%
Meinicke was negative in	11.04 to 10.35%

Table III shows the identical results obtained with all the tests used, according to the stage of the disease, as well as a summary of the results obtained.

Table IV shows the percentage of positives in each stage of the disease as well as in treated and untreated cases with each test employed. This is shown in Table V.

TABLE V

CASES	WASSERMANN (ORIGINAL)	SACHS-GEORGI WITH SERUM ACTIVE	INACTIVE	MEINICKE	BRUCK
Treated	52.08–53.82	57.0 –60.0	44.44–49.3	42.57–78.68	50.0 –52.08
Untreated	60.5 –66.6	73.73–87.65	28.4 –37.37	50.62–54.32	68.0 –71.6
Primary	49.27–50.72	56.52–58.0	40.58–47.82	47.82–55.52	47.87–50.72
Secondary	91.9 –93.7	91.9 –93.7	90.1 –91.9	91.9 –92.8	91.9 –92.8
Tertiary		89.27	See Table III		
Latent syphilis		68.0	(Identical results)		
Parasyphilis		66.6			

TABLE VI

THE SEROLOGIC RESPONSE OF EACH TEST TO THE 639 CLINICALLY POSITIVE CASES OF SYPHILIS

STAGE	WASSERMANN		SACHS-GEORGI WITH ACTIVE SERUM		SACHS-GEORGI WITH INACTIVE SERUM		MEINICKE		BRUCK		REMARKS
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	
Treated cases	155	133	172	116	142	146	140	148	150	138	
Untreated	54	27	71	10	30	51	44	37	59	22	
Primary	35	34	40	29	33	36	39	30	35	34	
Secondary	104	7	104	7	102	9	103	8	103	8	
Tertiary	49	7	49	7	49	7	49	7	49	7	
Latent syphilis	19	9	19	9	19	9	19	9	19	9	
Parasyphilis	4	2	4	2	4	2	4	2	4	2	
Total	420	219	459	180	379	260	398	241	419	220	
Percentage	65.72		71.83		59.31		62.28		65.57		

"Positive" includes all sera showing ++++, +++, ++ and +.
"Negative" includes ± and –.

In the order of positive results obtained in 639 clinically positive cases the tests stood (Table VI):

1. Sachs-Georgi with active serum and 2 per cent saline, 71.83 per cent.
2. Wassermann, 65.72 per cent.
3. Bruck, 65.57 per cent.
4. Meinicke, 62.28 per cent.
5. Sachs-Georgi with inactive serum and 0.85 per cent saline, 59.31 per cent.

If the positive results of the Wassermann reaction are taken as a standard, the other tests stand:

1. Sachs-Georgi with active serum and 2 per cent saline, 109.28 per cent.
2. Sachs-Georgi with inactive serum and 0.85 per cent saline, 90.23 per cent.
3. Meinicke, 94.70 per cent.
4. Bruck, 99.78 per cent.

The method proposed is of advantage:

1. As a control for the Sachs-Georgi test with inactive serum.
2. As a control for treatment.
3. In primary cases of syphilis.

BIBLIOGRAPHY

[1]Seelman: Am. Jour. Syph., 1920, iv, 157.
[2]Hecht, Hugo: Dermat. Wchnschr., 1922, lxxiv, 300.
[3]Traube: Ibid, p. 304.
[4]Liebermann: München. med. Wchnschr., 1917, p. 1462.
[5]Lecomte du Nouy: Jour. Exp. Med., 1922, xxxvi, 537.
[6]Neukirch, P.: Ztschr. f. Immunitätsforsch., 1920, xxix, 177.
[7]Merkl: Ztschr. f. Hyg., 1902, xxxix, 86.
[8]Joffick, S. D.: The Mechanism of the Sachs-Georgi Test and Its Relation to the Wassermann Test. (in press)
[9]Georgi, F., and Lebenstein, H.: Ztschr. f. Immunitätsforsch., 1922, xxxiii, 503.
[10]Bruck: Deutsch. med. Wchnschr., 1922, No. 25.
[11]Joffick, S. D.: Practical Suggestions for Performing the Sachs-Georgi Test. (in press).

A MECHANICAL METHOD FOR RECORDING SMALL VARIATIONS IN PRESSURE AND VOLUME*

By J. Craig Potter, M.D., Rochester, Minn.

IN laboratory work the need for an instrument to record low pressures and small variations of pressure or volume in fluids of low specific gravity is often met. The usual recorders are difficult to use because of the decrease in the writing amplitude as compared with the actual pressure change. In some special work involving small variations in pressure and in volume under low pressure an apparatus was developed which in my hands has given excellent results. The apparatus records directly in millimeters of pressure. In this way the pressure relations are easily visualized, the pressure may be

*Fellow in Surgery, The Mayo Foundation, Rochester, Minnesota.
Received for publication, January 29, 1924.

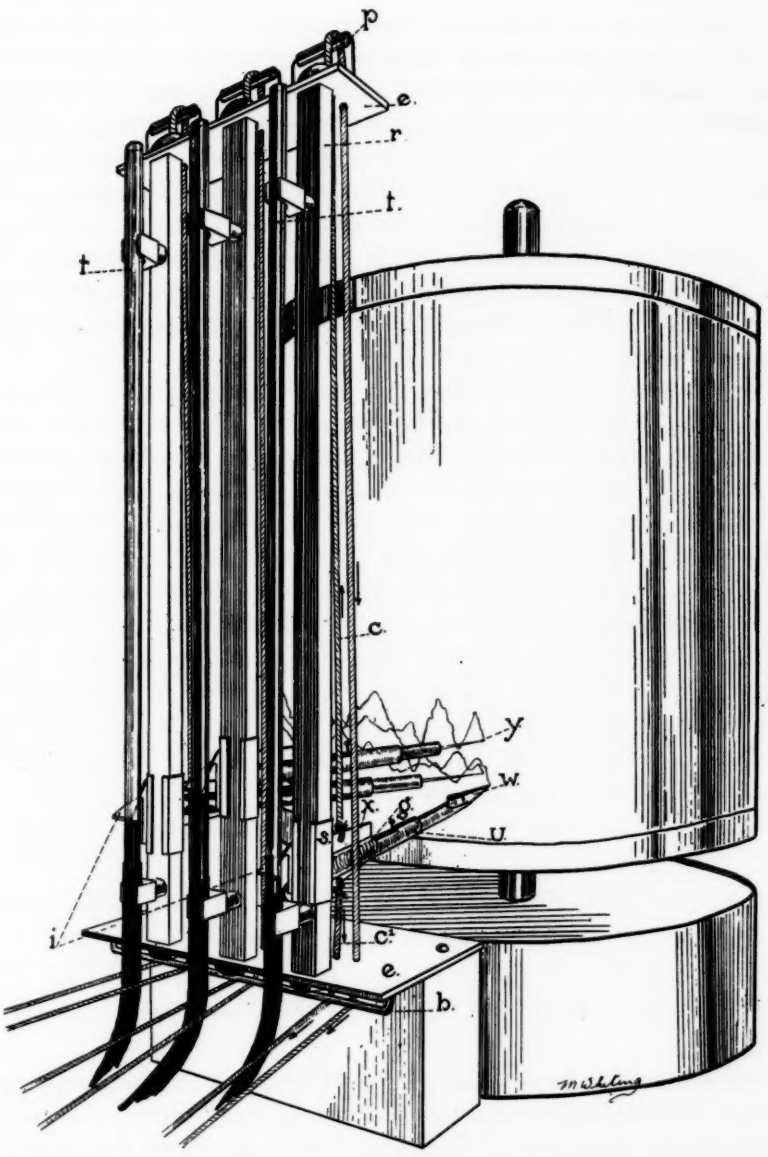


Fig. 1.—The apparatus consists of a standard made of three upright square brass rods (r) held in place by two end plates (e). In front of each rod is fastened a tube (t) which contains the fluid whose pressure is to be recorded. Each upright carries a square sleeve (s), on one side of which is soldered a thin brass strip (x). The tube end of the strip is narrowed to form an indicator (i) so that one may more easily follow the meniscus of the fluid. On the other end of the strip is soldered a small tube (u), in which is a writing point (w) for ink, or a stylet (y) for use on a smoked surface. The point is held constantly against the drum of the usual kymograph by a small spring (g) in the tube. A cord (c) attached to the strip (x) passes up over a glass pulley (p), down, around a bar (b) and out to one hand of the observer. Traction on this cord raises the writing point and indicator on the meniscus side. Another cord (c^1), attached also to the strip, passes down, around the bar and out to the observer's other hand. Pulling on the latter cord therefore, lowers the indicator. With one hand on each cord the upper meniscus of the fluid may be followed accurately. One person is required to operate each unit.

measured later, and knowing the diameter of the tube, the volume change may be computed.

The principle is the following of the meniscus of a fluid by means of a writing point controlled by hand. Man power thus gives the necessary energy to record a small change of pressure or volume in a glass tube of small bore. The size of the tube containing the liquid determines the delicacy of the movements, but a tube of less than 3 mm. inside diameter brings in capillarity which decreases the sensitivity of the pressure or volume change. The tracing may be made with either a stylet on a smoked drum, or, if recording several pressures, writing points with various colored inks may be substituted on white paper. To record synchronously more than one pressure it is only necessary to add a second or third unit, taking care that the points are bent to write close together.

The method is very sensitive and with a plethysmograph one may record the cardiac and respiratory waves in the animal, as well as other larger and slower pressure waves. The advantage of the apparatus is that one may make graphic records of low pressures and small variations of pressure and volume that cannot be recorded with the usual apparatus. The main disadvantage is that it is not mechanical. Although with training the personal equation is almost eliminated, there is always the reaction time difference between the actual and recorded pressure. Notwithstanding these disadvantages it is the best method I have tried for recording changes in the volume of organs such as the spleen, and for recording low pressures such as are found in the gall bladder and the common bile duct. Finally, it is probably as free from error as any of the methods which are not frictionless, such as the photographing of a ray of light.

CONCERNING THE STABILITY OF STOCK SALINE DILUTIONS OF ANTISHEEP HEMOLYSIN*

By Robert A. Kilduffe, B.A., M.A., M.D., Los Angeles, Calif.

THE majority of methods in common use for testing for complement-fixation in syphilis utilize an antisheep hemolytic system.

Whether one adopts a preliminary daily titration of complement, or of amboceptor or, as seems preferable, of both, in order to keep the hemolysin dose within limits which can be easily and accurately measured it is customary and convenient to prepare a stock dilution of amboceptor in normal saline.

A 1:100 dilution is conveniently prepared by the use of the following proportions:

Equal parts of hemolytic serum and glycerin	2 c.c.
Normal (0.85 per cent) saline solution	94 c.c.
5 per cent phenol in normal saline	4 c.c.

*Received for publication, January 29, 1924.

Such a dilution contains 0.2 per cent carbolic acid which effectually serves to prevent bacterial contamination unless this is gross in character and will keep for some time in the refrigerator.

Under ordinary circumstances, even in an active laboratory, this amount of hemolysin dilution will suffice for several weeks, at least; in a laboratory in which the number of daily tests is relatively small it will not need to be replenished for a correspondingly longer time.

When using the quantitative method of complement fixation described by Kolmer[1] in which the hemolysin dose averages 0.5 c.c. of 1:3,000 or 1:6,000 the life of the stock dilution is perceptibly lengthened. It becomes of some interest, therefore, to inquire as to the stability of such dilutions as affecting the frequency and amount in which they may be prepared.

In an active laboratory one hundred cubic centimeters of such a dilution will generally be consumed before any marked deterioration can take place; in a smaller and less active laboratory, however, such a dilution might last until such time as doubts of its potency and activity might arise.

The purpose of this note is to record the fact that, when kept in the refrigerator—except for the periods when in use—and when gross bacterial contamination is avoided, such dilutions have remained active, potent, and practically unchanged in titer—other than the changes due to variations in the activity of various complements—for a period of six months.

When gross bacterial contamination occurs, although the hemolysin is still active, a marked change in the titer occurs and as the amount of foreign protein in the test is undoubtedly a factor of importance, such bacterially contaminated amboceptors with a low titer are best discarded.

REFERENCE

[1]Kolmer, J. A.: A New Complement-Fixation Test for Syphilis Based upon the Results of Studies in the Standardization of Technic, Am. Jour. Syph., Jan., 1922, vi, No. 1.

A NOTE UPON THE STABILITY OF PRESERVED (GLYCERINATED) ANTISHEEP HEMOLYSIN*

By Robert A. Kilduffe, B.A., M.A., M.D., Los Angeles, Calif.

THE majority of methods in common use for the conduct of complement-fixation tests for syphilis make use of an antisheep hemolytic system and thus necessitate a constant supply of antisheep hemolysin.

Fortunately for facility in the conduct of the test, the preservation of hemolytic sera is entirely feasible and a common procedure, but while the usual texts upon the subject call attention to the fact that such sera are easily preserved in a stable and active condition for "some months," and while Kolmer[1] states that glycerinated serum remains remarkably constant in hemolytic titer for "at least a year" when kept in the refrigerator, one

*Received for publication, January 29, 1924.

fails to gain, in a cursory review of the literature, any exact idea as to the limit of time during which the hemolytic activity of serum may be maintained under ordinary conditions, and gets the impression that the length of time is somewhat sharply limited and not unduly prolonged.

The subject is of some practical importance from the standpoint of both the worker preparing his own hemolysin and those purchasing commercial products as influencing the frequency with which antisheep hemolysin must be prepared or purchased.

The preparation of antisheep hemolysin of high titer is such a relatively simple and inexpensive procedure that, if its preservation over long periods of time can be assured, there is little reason for the use of relatively low titer commercial preparations.

Methods for the preservation of hemolytic sera are numerous and varied but the simplest and most efficacious is the use of chemically pure glycerin as advocated by Kolmer[2] and Clock and Beard.[3]

Glycerin in the proportion of 50 per cent is not only a satisfactory antiseptic and preserves the serum clear and uncontaminated by bacteria, even when the serum is preserved in bulk and frequently tapped, but also, by reason of its hygroscopic qualities prevents molecular changes and thus preserves the hemolytic titer.

As is well known, the titer of a hemolytic serum is higher before than after inactivation by reason of the destruction of the native complements and also of thermostabile hemolysins; as is equally well known a similar destruction occurs on standing. Nevertheless, it has seemed useful and advisable to heat hemolytic serum for thirty minutes at 56° C. before preservation as this procedure may serve to destroy vegetative forms of bacteria which might be present thus leaving to the antiseptic action of the glycerin the destruction of only those forms which may gain entrance thereafter.

Following the inactivation of the serum its final titer is determined and an equal quantity of chemically pure glycerin added, the two well mixed, and ampuled in 2 c.c. ampules, each thus containing 1 c.c. of serum and so forming a convenient starting point for the preparation of stock saline dilutions.

In 1916 an antisheep hemolysin was prepared and preserved according to the method outlined above. The initial titer was 1:20,000.

In 1917, when the writer entered the army, the unused portion of this serum was placed in the refrigerator. After discharge in 1919 the serum was used for the performance of complement-fixation tests with entire satisfaction. It was later shipped by freight from Philadelphia to Pittsburgh and thus, for some little time, was exposed to summer temperature in spite of which it was still active in high dilutions.

Some of the ampules were kept in the refrigerator until 1920 and again tested and found to have a titer of 1:18,000. Allowing for variations in the activity of various complements the titer is thus seen to be practically unchanged after four years of preservation.

A few ampules were set aside for still further preservation and in January, 1923, were found entirely suitable for use with the quantitative

complement-fixation test described by Kolmer[4] giving complete hemolysis in a dilution of 1:16,000 with 0.3 c.c. of 1:30 complement.

Preservation by the use of equal parts of chemically pure glycerin was satisfactorily achieved, therefore, for a period of seven years.

REFERENCES

[1]Kolmer, J. A.: A Study of Methods for the Preparation and Preservation of Hemolysins, Am. Jour. Syph., 1920, iv, 3.
[2]Kolmer: Ibid.
[3]Clock, R. O. and Beard, S. D. Preservation of Antisheep Hemolytic Amboceptor in Glycerol, Jour. Infect. Dis., 1917, xxi, 404. Quoted by Kolmer.[1]
[4]Kolmer, J. A.: A New Complement-Fixation Test for Syphilis, Am. Jour. Syph., 1922, vi, 1.

A METHOD FOR THE RAPID FIXATION OF TISSUE SECTIONS*

By Edward F. Lane, New Britain, Conn.

THE time required for the diagnosis of tissue sections has always been a bugbear to the surgeon with a case of suspected malignant disease, and at best the frozen section is inadequate and often not wholly reliable. But as quicker methods for preparing reliable paraffin sections are being worked out, the one-time ideal method is fast becoming obsolete.

The following method has given very satisfactory results in our laboratories and at the same time is quicker than any other paraffin method which has come to my notice. A diagnosis can be made in eight hours from the time of removal of tissue. Pieces of tissue are cut with sufficient surface to allow an adequate study of the tissue under observation, but must not be more than two millimeters thick. These are threaded on a length of sewing cotton with a fine needle. A glazed paper tag, just thick enough to remain firm, is also threaded on the cotton. This has the identification mark of the specimen written on it in pencil. In the case of a number of specimens being run through at the same time they can all be threaded on the one thread with the number tags in front of each specimen.

Eight covered jars are necessary for the method; also two paraffin containers for use in the paraffin oven. This oven is to be kept at about 58° C. The first three jars contain 95 per cent alcohol, the fourth acetone, the fifth oil of cedar leaves, the sixth and seventh chloroform, and the eighth a saturated chloroform solution of paraffin. Each of the containers in the paraffin oven contains paraffin of melting point about 55° C.

The fresh section is placed in the first jar without preliminary fixation in formalin or other reagent. After remaining for half an hour, it is transferred to jar number two for one-half hour. It remains in jar number three for two hours, and is then passed through the other jars in succession, remaining fifteen minutes in each. From there it is put in first one and then the other of the paraffin containers, remaining fifteen minutes in each.

Sections are taken from the second paraffin container and made into

*Received for publication, Feb. 5, 1924.

From the Laboratories of the Departments of Pathology and Experimental Medicine, New Britain General Hospital.

small blocks, in the usual manner. These are put in the refrigerator to harden, a process taking less than half an hour to secure a very hard block. From this point on the sections are cut, transferred to egg albumen smeared slides, are heated in the paraffin oven for half an hour and then stained by the usual hematoxylin and eosin method.

The method is also being used in our laboratory for routine work. In this case the specimens which come from the operating room are placed in saline solution until late in the afternoon. The gross examination is then made and the sections threaded onto one or more pieces of cotton, depending on the number of tissues to be examined. They are run through the first two alcohols as usual and are then left in the third alcohol until morning. In the morning the foregoing technic is employed. This allows us to report on all tissues the day following removal, unless special staining methods are required.

AN ANESTHESIA CHAMBER FOR LARGE ANIMALS*

By Minas Joannides, M.D., Minneapolis, Minn.

IN the course of experimental work on dogs I have at times experienced considerable difficulty in anesthetizing the animals. During the stage of induction the animals often struggled so that it required two persons to hold

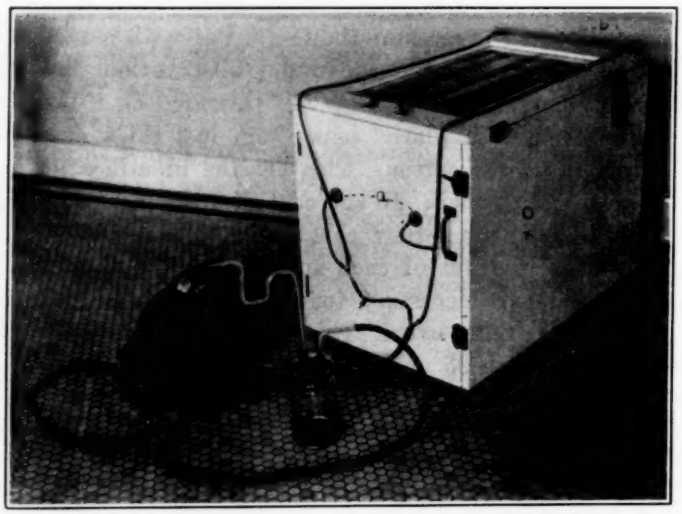


Fig. 1.—Closed chamber showing arrangement of the tubing.

them. To avoid requiring extra help, and at the same time be more humane, I have devised an etherizing chamber in which the animal may be anesthetized by pumping in etherized air.

The chamber is divided into two compartments, so that two dogs may be anesthetized at one time. It becomes practically airtight when closed

*From the Laboratory of Experimental Surgery, University of Minnesota Medical School. Received for publication, February 10, 1924.

and fastened with sash locks. The inner walls may be padded, so that in case the animal struggles it will suffer no injury. The box is, approximately, thirty-one inches long, twenty-one inches wide and twenty-four inches deep. It may be opened from above or from the front. It has a glass window on top and one on the side opposite the door, thus affording ample light. Ether is introduced through holes, a, b, x, y, each being one and one-quarter inches in diameter. These are closed with rubber stoppers perforated for glass tubing from one-half to two centimeters in diameter. The tubes are connected with a bottle containing ether and this, in turn, to a bellows which is manipulated by the foot. Rubber tubing is used for making connections

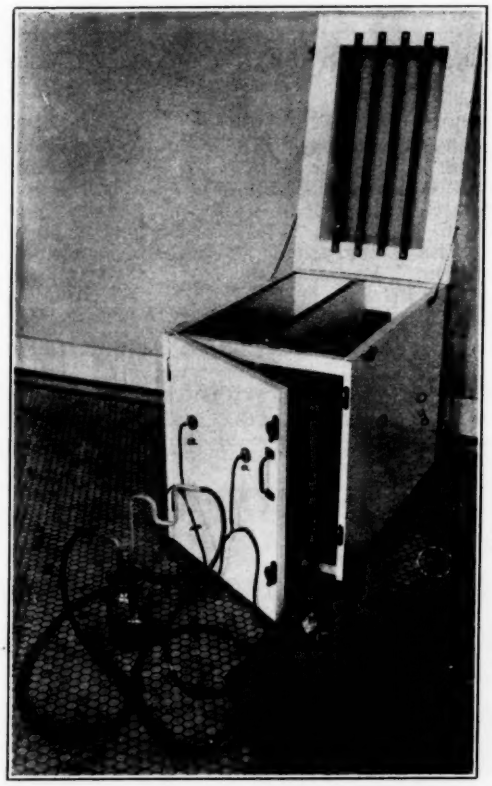


Fig. 2.—Both doors open, showing interior of the chamber.

The glass tubing ("c" in Fig. 1) is immersed in the ether and air thus saturated with ether is introduced into the chamber. After complete anesthesia is established, if desirable, one may easily remove the bottle and use it for maintaining anesthesia. It is then connected to two catheters inserted into the mouth and nostril respectively. The excess air intake is controlled with a towel wrapped around the dog's nose. Pumping etherized air through the bottle enables one to easily maintain any desirable degree of anesthesia and to perform an operation with little additional help.

By using this method the operator can administer the anesthetic and still perform an experiment under aseptic conditions.

The Journal of Laboratory and Clinical Medicine

VOL. IX ST. LOUIS, MO., JUNE, 1924 No. 9

Editor-in-Chief: WARREN T. VAUGHAN, M.D.
Richmond, Va.

ASSOCIATE EDITORS

DENNIS E. JACKSON, M.D. - - CINCINNATI
PAUL G. WOOLLEY, M.D. - - DETROIT
J. J. R. MACLEOD, M.B. - - - TORONTO
ROY G. PEARCE, M.D. - - AKRON, OHIO
W. C. MACCARTY, M.D. - ROCHESTER, MINN.
GERALD B. WEBB, M.D. - COLORADO SPRINGS
VICTOR C. MYERS, PH.D. - - NEW YORK
MARSH PITZMAN, M.D. - - - ST. LOUIS
RUSSELL L. HADEN - - ROSEDALE, KAS.

Official Organ of the American Society of Clinical Pathologists

Contents of this Journal Copyright, 1924, by The C. V. Mosby Company—All Rights Reserved

Entered at the Post Office at St. Louis, Mo., as Second-Class Matter

EDITORIALS

Gas Requirements of the Tubercle Bacillus

THAT the tubercle bacillus is an obligatory aerobe is well known. Of late some attention has been given to its other gas requirements, and to its behavior when deprived of oxygen.

Webb, Ryder, and Gilbert have reported the loss of virulence which occurs when tuberculous animal tissues, especially lymph nodes, are incubated in normal salt solution at about body temperature. In a fresh state such tissues generally produce the usual acute tuberculosis when planted under the skin of healthy guinea pigs. After a few days' incubation they produce a chronic tuberculosis, which may last more than two years. After being incubated for a week or two they produce no disease at all and the bacilli appear to be dead. At ice-box temperature the phenomenon occurs very slowly if at all.

Similar facts observed several years ago by Bartel, in the case of tubercle bacilli introduced into normal lymph nodes and then incubated, were attributed by him to some specific bactericidal virtue of lymphoid tissue. However, Webb and his associates obtain the same results with pieces of tuberculous liver, and have always thought it probable that they are due to

deprivation of oxygen analogous to that which occurs at the bottom of culture tubes of broth to which bits of tissue have been added.

Recently C. H. Boissevain of Colorado Springs has made parallel experiments with emulsions of tubercle bacilli from cultures, the results of which have not yet been published. The tubes containing the salt solution emulsions are placed in pyrogallic jars and incubated, and the attenuation occurs at about the same rate as in incubated tissues, while in the ice box it is far slower. Controls not deprived of oxygen remain virulent for a long time.

It is possible that the phenomenon is more complex than it appears. Corper, Lurie, and Kretschmer have found the juices of incubated tissues bactericidal to tubercle bacilli under experimental conditions which make it improbable that the effect is a simple reduction. In the experiments with emulsions the bacilli may produce substances toxic to themselves, including, as Corper suggests, carbon dioxide.

Corper, Gauss, and Rensch, following up some observations of Wherry and Ervin, have reported that tubercle bacilli are prevented from growing by a carbon dioxide concentration equivalent to that which prevails in human tissues (5-6 per cent gas volume), and are killed by a few weeks' exposure to an atmosphere containing 12-15 per cent carbon dioxide. They also report that the bacillus will not grow in the complete absence of carbon dioxide.

In studying this point Boissevain finds that cultures on glycerine agar incubated in sealed jars containing some 40 per cent potassium hydroxide solution are inhibited; however, if glycerine broth cultures are placed in the same conditions they grow vigorously. The failure to grow in the first instance is quite clearly due to the drying which accompanies the absorption of carbon dioxide by this means.

Oxygen, in fact, appears to be the only gas which is essential to the normal life and growth of the tubercle bacillus. Cultured in glycerine broth in an atmosphere of pure oxygen it grows well, but the growth instead of remaining on the surface, as it does in air, tends to sink to the bottom of the flask. This presents a suggestive analogy to some of Rosenow's observations on streptococci. He finds that in tall tubes of broth with a piece of tissue at the bottom, these organisms grow at the level of optimum oxygen tension, which varies for different strains. In mixtures of air and nitrogen Boissevain finds that the growth of the tubercle bacillus in glycerine broth varies directly with the percentage of air, and apparently ceases when this is reduced to about 20 per cent.

If anything is to be hoped for in the prevention and treatment of tuberculosis by vaccination in the traditional sense, asphyxiation may possibly be a favorable method for preparing killed or attenuated suspensions. Bartel from his early experiments was led to believe that animals which received tubercle bacilli from incubated tissues showed changes in their subsequent resistance to virulent infection, and the Colorado Springs investigators have some observations which seem to justify this opinion. However, the effectiveness of the immunity changes which result, even from actual healed or well resisted first infection with tuberculosis, is so dubious, that we probably should not hope too much from such procedures. At any rate the pres-

ent tendency seems to be to let this field lie fallow for a while, and to turn our attention to the chemistry of host and parasite, in the hope of gaining a better understanding of what happens in disease, and of how we may best intervene to prevent or arrest it.

REFERENCES

Webb, Ryder, and Gilbert: Amer. Rev. Tuberc., Feb., 1916, i, 693.
Webb, Ryder, and Gilbert: Tr. Assn. Amer. Phys., 1918.
Webb, Ryder, and Gilbert: Tr. Nat. Tuberc. Assn., 1921, xvii, 288.
Webb, Ryder, and Gilbert: Amer. Rev. Tuberc., July, 1921, v, 388.
Webb, Ryder, and Gilbert: Tr. Nat. Tuberc. Assn., 1923, xix, 290.
Bartel and Neumann: Centralbl. f. Bakt., 1905-6, Orig. xli, 518.
Bartel and Neumann: Ibid, 1909, xlviii, 657.
Bartel: Wien. klin. Woch., April 19, 1906, xix.
Corper, Lurie, and Kretschmer: Tr. Nat. Tuberc. Assn., 1923, xix, 285.
Corper, Gauss, and Rensch: Tr. Nat. Tuberc. Assn., 1921, xvii, 312.
Wherry and Ervin: Jour. Infect. Dis., 1918, xxii, 194.

—G. B. W. (C. T. R.)

The Uses and Limits of Vaccine Therapy

THE title applies to a symposium on the clinical aspects of vaccine therapy appearing in the April, 1924, number of the Proceedings of the Royal Society of Medicine. A recent editorial in these columns called attention to the unsatisfactory state of vaccine therapy. By this we do not refer so much to prophylaxis. Of the many remedies used dogmatically, without consideration for scientific principles, vaccines in general find a place high in the list. Dosage is as a rule purely empiric and the route of administration is chosen primarily for facility and because it alleviates the necessity of careful graduation of the dose. Stock vaccines are selected according to number, and the physician often finds himself unable to state offhand what bacteria are represented in his favorite stock, much less the relative proportions thereof. Probably also there are too many stock vaccines. We refer particularly to the mixed vaccines representing five or six different microorganisms. One species more or less, provided it is not the specific microorganism responsible for the malady in question, in all probability makes but little difference. On the other hand, the stock vaccine probably often contains an insufficient number of strains representing any one organism. There is some evidence that certain strains of one germ produce better results as a vaccine than do other strains of the same bacterium. Little consideration is often given to this observation.

Autogenous vaccines, except where their preparation or administration is supervised by a student of vaccine therapy interested from the experimental point of view, are often made incorrectly—started after rapidly growing secondary invaders have overgrown the true pathogen or made with inappropriate culture media, or technic.

An occasional case of one disease or another appears to have acquired brilliant results from vaccine therapy. His physician, a true optimist, continues the same method without success in case after case, forgetting his failures and recalling only his signal achievement. As a whole, few general practitioners of medicine read any literature on vaccine therapy other than the elaborate brochures distributed by the various manufacturers of vaccine.

This unsatisfactory situation is well understood, and many immunologists are working toward standardization of technic and formulation of scientific rules for treatment. The promising work of Sir Almroth Wright has already been referred to.[1] In the meantime we may well take inventory of what has been accomplished with vaccine treatment and learn in what cases we may anticipate success or failure.

With regard to dosage, Wright, who opened the symposium referred to, emphasizes certain important facts. A healthy man gives a certain response to the injection of a large dose of bacteria. An unhealthy man with a large number of the bacteria already on his economy, gives a like response to a much smaller injection of bacteria. An infected man therefore requires a smaller dose of vaccine for certain results than does a healthy man. It becomes necessary then to determine the dimensions of his infection in order that the dose of vaccine may be made proportionate. If the dimensions of the infection are increasing, a time will come when the bacterial concentration already existing in the system becomes too great to elicit satisfactory immunity response. In such a case protective substances must be provided from the outside, with passive rather than active immunization. These principles briefly summarize the rationale of Wright's new technic of intravenous dosage for elicitation of the epiphylactic response.

Wright doubts the universal specificity of antibodies and points out that their concentration in the blood increases to an optimum almost instantaneously after intravenous inoculation with the proper quantum of vaccine. The germ used to obtain this antibody response need not be the specific bacterium responsible for the infection. The fact that about ten days are normally required for the production of specific protective substances indicates the existence of more than one class of antibody, one specific and another nonspecific. The group of specific antibodies appears to be of more importance in prophylaxis while the nonspecific substances are probably of greater value in treatment.

Assuming that the crisis in pneumonia is due to the ultimate accumulation of sufficient pneumococci to produce an optimal or critical dose of antigen, Wright suggests that if we could discover the proper proportionate dose necessary on the first or second day of an attack of pneumonia, its administration might produce an immediate immunizing response with immediate crisis. This hypothesis is most interesting and while such may eventually be accomplished, it is scarcely practical with present methods.

Dr. A. P. Beddard, like Wright, emphasizes the regulation of dosage with regard to the bacterial concentration of the infection, and agrees that the logical time for the administration of vaccine would be before the constitutional symptoms have become severe or after they have passed their height. In the latter case, that is, during convalescence, vaccines would rarely be indicated, although in unresolved pneumonia, arthritis, bronchopneumonia, etc., there might occasionally be some such indication.

He has repeatedly seen stock pneumococcus vaccine in dosage of 25 to

[1]Jour. Lab. and Clin. Med., May, 1923, viii, 549.

50 million administered subcutaneously in pneumonia, within the first 48 hours, without apparent beneficial result. However, it should be pointed out that this does not answer Dr. Wright's suggestions, since there is no evidence that the dose was standardized according to Wright's technic, nor was the treatment given by the route recommended.

Beddard finds serious limitations to the value of vaccines in chronic infections. The more chronic the case the more difficult it becomes to stir up the immunity mechanism. Thus while active colon bacillus pyelitis is readily curable with the use of vaccines, the chronic carrier of colon bacillus in the urinary tract remains so in spite of vaccination. He finds distinct limitation also to the therapeutic value of vaccines in infections of epithelium-lined cavities, such as the alimentary tract, the urethra, the urinary tract, the Fallopian tubes and to a less extent the respiratory tract. If in addition such an infection is chronic, cure by vaccine, however long or skillfully given, is unlikely. If there is any interference with drainage of the infected material, treatment will be unsuccessful until the mechanical obstruction has been removed.

While observing good results from mixed stock or autogenous catarrhal vaccine in the prevention of coryza, Beddard found that the chronic carriers of upper respiratory tract infection, those afflicted with one cold after another, did not respond as satisfactorily.

Beddard enumerates as avoidable causes for failure in this form of treatment, that (1) the correct pathogenic organism may not have been isolated, (2) the dose interval selected may be incorrect, (3) too much is expected from the method, too few doses being given, and that (4) other forms of treatment such as establishment of drainage, removal of infectious foci, etc., are too often neglected.

Sir William Leishman discusses the various methods of preparing bacterial vaccine such as sensitized vaccines, serovaccines, lipovaccines, detoxicated vaccines, diaplyte-vaccines, and concludes that none of these special types have shown any striking advantage over the older methods of simple sterilization by heat or with an antiseptic. He finds little superiority for autogenous vaccines over the proper stock vaccines provided the latter have been carefully chosen for their superior antigenic properties.

Beddard prefers autogenous vaccines to stock vaccines. He finds sensitized vaccines less toxic but doubts whether they offer any advantages commensurate with the greater trouble and expense of preparation.

As usual, we observe, here, a difference of opinion as to the relative values of stock and autogenous vaccines. Autogenous vaccines, when promptly and properly prepared appear on the whole to have a slightly superior value over stock vaccines. However, we must bear in mind that the prophylactic use of stock vaccines, in smallpox, typhoid and the paratyphoid fevers has been highly successful. During the late war, Cecil and his collaborators observed apparent protection against the various types of pneumococcus infections with the use of stock vaccines.

In the presence of acute infection, the time lost in the preparation of autogenous vaccines is often a distinct disadvantage. Stock gonococcus and

colon bacillus vaccines are practically as effective as are the autogenous preparations. Kolmer believes that "as a general rule stock vaccines should be polyvalent in order to include strains that may be serologically distinct. While autogenous vaccines are to be preferred in the treatment of disease provided they are properly prepared, stock vaccines possess equal powers for nonspecific immunization and serve useful purposes."

Dr. Richard Armstrong finds little actual difference between the action of sensitized vaccine and nonsensitized or raw vaccine. He believes that the former carries an antibody charge which gives an immediate passive immunizing effect which becomes exhausted within twenty-four hours. This is later followed by the development of active immunity as is the case with nonsensitized vaccines.

"There are fashions in treatment," says Norman McCaskie, "and few of us who have been in practice for over twenty years can look back with quite clear consciences on our attitude towards them. Vaccine therapy today is the fashion of the moment. It has captured the general public; our patients expect it, and reproach the man who does not employ it as being behind the times. This reacts on us, and there is hardly a bacterial infection in which we feel that we have exhausted the last means of treatment unless we make use of a vaccine. It is fatally easy—and very profitable—but it lends itself to exploitation. There will be a reaction, and a prejudice will arise against a method of treatment, which though yet in its infancy and hardly as yet of general application, holds great promise for the future."

—*W. T. V.*

BOOK REVIEWS

(Books for Review should be sent to Dr. Warren T. Vaughan, Medical Arts Building, Richmond, Va.)

*Fasting and Undernutrition**

INANITION is studied not only in those phases which we customarily have in mind when discussing the phenomenon, but also in its relationship to other closely allied conditions. Chief among the latter is that physiologic inanition which occurs in hibernation, in metamorphosis as from the tadpole state to that of the frog, and in breeding, particularly with the salmon, whose reproductive instinct is so strong that after reaching the spawning territory, the fish often dies from starvation and exhaustion. The study of hibernation is comprehensive and makes unusually interesting reading. Following physiologic inanition, experimental inanition is discussed in its chemical physiologic, biophysical and biochemical phases. Under partial inanition the effect of restriction in one or more of the food elements, including the vitamines, is discussed. Underfeeding and undernutrition in general are classified under chronic inanition and the practical features of such partial starvation are exemplified in the experiences of the Central Powers during the World War. An addendum contains a comprehensive bibliography.

*Fasting and Undernutrition. By Sergius Morgulis. Professor of Biochemistry. University of Nebraska College of Medicine. Cloth. Price, $5.00. Pp. 407. E. P. Dutton & Company, New York, 1923.

*War Blindness at St. Dunstan's**

THE author has given us in a most attractive form a concentrated history of practically every man in the British Army who was blinded in the World War. He has presented the work of St. Dunstan's from a purely medical point of view, and when studied in connection with Sir Arthur Pearson's work at St. Dunstan's called "Victory over Blindness" it is especially interesting.

The 1833 cases are divided into the traumatic and nontraumatic types. We can but be impressed with the large percentage which belong to the latter type (26 per cent), and its tremendous bearing on the importance of careful examinations of the recruits, especially for syphilis which is chiefly responsible for this large percentage. Probably the most striking fact brought out in regard to the traumatic cases is the entire absence of sympathetic inflammation following the destruction of one eye by shell splinters or bullet wounds. On the other hand, he reports several cases following some operative procedure done on the wounded eye; this further corroborating the fact that sympathetic disease is a particularized form of sepsis.

The author has an exceedingly interesting chapter on disability pensions, and in this he speaks at length on the question of blindness associated with syphilis arising in soldiers during the period of the war.

The one chapter alone on reeducation of the blind makes the book well worth while.

Bergey's Manual of Determinative Bacteriology†

THE Committee of the Society of American Bacteriologists of which Dr. Bergey is chairman and Francis O. Harrison, Robert S. Breed, Bernard W. Hammer and Frank M. Huntoon are members, has compiled a manual for the identification of bacteria. No thoroughly satisfactory manual of this type has been written since Novy's and Chester's were published many years ago. In the interval much new knowledge has been gained; new microorganisms have been described, new methods of study and culture have been developed and classifications have been rearranged and the nomenclature has been altered. It is to be hoped that the nomenclature which appears in Bergey's manual will become standard, particularly since it is essentially a descriptive terminology. Until it comes into general use we may anticipate some unavoidable confusion. The classification of bacteria is based upon morphologic, cultural, biologic and chemical characteristics. The volume should well fulfil its purpose, which is chiefly the standardization of general bacteriologic technic and classification.

---

*War Blindness at St. Dunstan's. By Sir Arnold Lawson, K.B.E., M.D., F.R.C.S., L.R.C.P., Senior Ophthalmic Surgeon and Lecturer on Ophthalmic Surgery, Middlesex Hospital; Late Surgeon, Royal London Ophthalmic Hospital, etc. Cloth. Price, $2.50. Pp. 148. Henry Frowde and Hodder and Stoughton, London. 1922.

†Bergey's Manual of Determinative Bacteriology. A Key for the Identification of Organisms of the Class Schizomycetes. Arranged by a Committee of the Society of American Bacteriologists: David H. Bergey, Chairman, Francis C. Harrison, Robert S. Breed, Bernard W. Hammer, Frank M. Huntoon. Cloth. Pp. 442. Williams and Wilkins Company, Baltimore, 1923.